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(54) Title: CELLULAR ADJUVANT

(57) Abstract

    This invention relates to a method of maturation of dendritic cells *in vitro* a serum–free medium, and to methods of treatment using the mature dendritic cells.

CELLULAR ADJUVANT

This invention relates to a cellular adjuvant and to a method of production thereof. In particular, the invention relates to activation of dendritic cells to mature, functional dendritic cells for use as a cellular adjuvant in treatment of neoplastic disease.

BACKGROUND OF THE INVENTION

The recent identification of tumour-related cytotoxic T lymphocyte (CTL) epitopes together with reports of clinical anti-tumour responses by adoptive transfer of autologous peptide-specific CTL has raised the possibility that peptide-based vaccination strategies may be useful for cancer treatment (Boon et al, 1994; Kawakami et al, 1995). The outstanding ability of dendritic cells (DC) to function as antigen-presenting cells (APC) makes them attractive as potential cellular adjuvants for activating and expanding peptide-specific anti-tumour CD8$^+$ CTL (Bakker et al, 1995; Bhardwaj et al, 1994; Macatonia et al, 1991).

However, the lack of lineage-specific surface markers and the multitude of ways of purifying or growing DC have made it difficult to characterize these cells definitively. DC are usually identified by their typical morphology, characterized by the presence of dendrites or membrane processes, and their characteristic surface antigen expression pattern (positive for CD1a, CD11c, CD40, CD80, CD83, CD86, HLA-A,B,C and HLA-DR, and negative for mature leukocyte markers of other lineages) (Macatonia et al, 1991; Szaboles et al, 1995; Caux et al, 1992).

To obtain DC for clinical application, a number of sources and culture methods has been proposed. DC can be purified from peripheral blood (Steinman and Young, 1991). They can also be grown in culture from peripheral blood mononuclear cells (PBMC) in the presence of cytokines such as granulocute/macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) (Romani et al, 1994;

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Inaba *et al*, 1992). Another technique for growing DC involves harvesting CD34⁺ cells from umbilical cord blood, bone marrow or cytokine-mobilized peripheral blood progenitor cells and culturing these cells in GM-CSF and
5   tumour necrosis factor-alpha (TNF-α) with or without IL-4 (Szabolcs *et al*, 1995; Caux *et al*, 1992; Romani *et al*, 1994; Santiago *et al*, 1992; Mackensen *et al*, 1995; Siena *et al*, 1995; Herbst *et al* , 1996; Strunk *et al*, 1996). To increase yields, the addition of stem cell factor (SCF)
10 either alone or together with a cocktail of cytokines (IL-1b, IL-3, IL-6, and erythropoietin) has been tried (Szabolcs *et al*, 1995; Mackensen *et al*, 1995; Santiago Schwarz *et al*, 1995).

Hitherto, none of these methods has proved
15 successful in providing a purified population of mature, functional DC in sufficient numbers for clinical use, although clinical trials using DC from PBMC have commenced. Methods of expanding the DC population in culture have therefor been sought.
20   Methods of growing DC usually utilize either foetal calf serum (FCS) (Szabolcs *et al*, 1995; Caux *et al*, 1992; Mackensen *et al*, 1995; Siena *et al*, 1995) or pooled human serum (Santiago Schwarz *et al*, 1992; Siena *et al*, 1995; Santiago Schwarz *et al*, 1995) in the culture medium.
25 However, the use of serum is undesirable for several reasons. Given that both peripheral blood DC precursors and Langerhans cells (LC) take up and process soluble antigens (Sallusto *et al*, 1995; Stossel *et al*, 1990), DC that differentiate from cultured CD34⁺ cells would be
30 expected to take up and process proteins derived from serum present in the medium. Indeed, when DC cultured in the presence of FCS were used to stimulate CTL *in vivo*, an overwhelming generation of cytolytic activity against bovine antigens was observed (Porgador and Gilboa, 1995).
35 Donor serum may also constitute an infectious risk for patients if DC are to be used clinically, and the use of xenogeneic serum is usually considered to be undesirable.

Finally, serum contains growth factors, and thus its presence might interfere with the study of the role of cytokines in DC differentiation and maturation, even though this may not be of great relevance to ultimate production of a cellular adjuvant for clinical use.

We have now developed a method to generate DC which function as potent APC for both allogeneic T lymphocytes and autologous, peptide-specific CTL, using CD34⁺ progenitors under serum-free conditions. To our knowledge we are the first to show that DC can be generated from CD34⁺ progenetors in the absence of the stem cell-expanding cytokines stem cell factor, IL-3, flt-3L or other expensive additives such as TGF-β.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a method of inducing the maturation of dendritic cells *in vitro*, comprising the step of culturing mononuclear cells in a serum-free medium in the presence of a Type I interferon following an initial phase of growth in the presence of GM-CSF, TNF-α and IL-4. Preferably the mononuclear cells are CD34⁺ haematopoietic progenitor cells.

The cells which are cultured may be peripheral blood progenitor cells, for example obtained by leukapheresis, or bone marrow progenitor cells. More preferably the starting cell population is also enriched for CD34⁺ cells prior to culture.

Preferably the cells are cultured for 14 to 35 days, more preferably from 14 to 28 days, and even more preferably for 14 to 17 days.

Preferably GM-CSF, TNF-α are present in the culture medium for the whole period of culture, and preferably the Type I interferon is present commencing at day 13 of culture. Preferably IL-4 is present commencing at day 6, or is present for the whole period of culture.

Any Type I interferon may be used, for example interferon-α, interferon-β, interferon-ω, or consensus

interferon (Infergen; Amgen, Inc., Thousand Oaks, California). Preferably the interferon is IFN-α2a, IFN-α8 or IFN-β.

5   If peripheral blood mononuclear cells are to be used, it may be advantageous to pre-treat the patient or animal from which the cells are to be obtained with a cytokine such as G-CSF or GM-CSF prior to leukapheresis in order to mobilise bone marrows stem cells into the circulation. Enrichment of between 10- and 1000-fold of

10   stem cells in the circulation is desirable. In the absence of such priming, there may be insufficient stem cells in the circulation, and bone marrow cells are then preferably used.

      Optionally the cells may also be cultured in the

15   presence of stem cell factor (SCF). Where SCF is used, it is preferably present throughout the culture period.

      Maturation of the dendritic cells may be monitored using morphological markers such as the presence of dendritic processes at the cell surface, and the

20   presence of immunological markers such as CD40, CD80, CD83, CD86, and CMRF44.

      The matured dendritic cells produced by the method of the invention are non-phagocytic, and have potent antigen-presenting activity. They are capable not only of

25   stimulating multiplication of allogeneic lymphocytes in the mixed leukocyte reaction (MLR), but also of stimulating autologous, peptide-specific cytotoxic T lymphocytes in the mixed leukocyte peptide culture assay system (MLPC).

      Therefore in a second aspect the invention

30   provides a method of enhancing the antigen-presenting capacity of dendritic cells, comprising the step of exposing dendritic cells to a Type I interferon. Any Type I interferon can be used, as described above. Interferon can be administered *in vivo*, in order to activate either

35   the recipient's own resident dendritic cells, including Langerhans cells or other tissue antigen-presenting cells, or antigen-presenting cells which have been infused or

injected and are subsequently activated *in vivo* by systemic administration of interferon. Where interferon is administered *in vivo* it may be delivered at the site of dendritic cell administration, as set out below or may be
5 administered in such a way as to spread systemically through the body. The interferon it may be given in conjunction with other cytokines which induce proliferation of DC; for example interferon may be given in conjunction with, ie sequentially or simultaneously with, GM-CSF or
10 GM-CSF and IL-4. Preferably dendritic cells are exposed to interferon *in vitro*, as described above.

In a third aspect, the invention provides a method of preparation of a cellular adjuvant for treatment of a neoplastic disease or a disease caused by an
15 infectious agent, comprising the steps of maturing dendritic cells *in vitro* as described above, and exposing the matured dendritic cells to an antigen derived from the neoplastic cells (*ie.* a tumour-associated antigen) or from the infectious agent, respectively.

20 The infectious agent may be a bacterium, a virus, a yeast or a parasite.

The antigen may be a protein, peptide, polysaccharide, or nucleic acid. The dendritic cells may be transfected with the antigen, or may be incubated *in*
25 *vitro* with the antigen.

The cellular adjuvant of the invention may be used in the treatment of a neoplastic disease, in order to provoke or stimulate an anti-tumour response. Thus the invention provides a method of treatment of neoplastic
30 disease, comprising the step of administering an effective dose of the cellular adjuvant according to the invention to a patient in need of such treatment. As an alternative to transfection of dendritic cells with antigen or incubation of dendritic cells with antigen in culture, matured
35 dendritic cells according to the invention may be administered to the patient together with the antigen. Any suitable route of administration may be used, for example

subcutaneous, intravenous, intratumour, or intra-lymphatic. Preferably the dendritic cells are autologous, *ie.* from the patient. The neoplastic cell antigen may also be derived from the patient's own tumour cells.

5   Similarly the cellular adjuvant of the invention may be used in the treatment of disease caused by an infectious disease, using an antigen derived from the infectious agent as discussed above.

The method of the invention may also be used to
10   generate clones of activated tumour-associated T cells. Thus, in another aspect the invention provides a method of producing a population of activated tumour-associated T cells, comprising the steps of harvesting dendritic cells and lymphocytes from peripheral blood or bone marrow of a
15   patient suffering from a tumour, maturing dendritic cells according to the method described above, and culturing the dendritic cells and the lymphocytes, either separately or together, in the presence of a tumour-associated antigen, and optionally in the presence of a cytokine. Preferably
20   the cytokine is a type I interferon, more preferably interferon-α.

This aspect of the invention also provides a method of treatment of a patient suffering from a tumour, comprising the step of administering an effective dose of
25   dendritic cell-activated, tumour-associated T cells to a patient in need of such treatment. Alternatively, this aspect of the invention provides a method of treatment of a pathological condition, comprising the step of administering an effective dose of mature dendritic cells
30   having the functional and antigenic characteristics as defined herein to a mammal in need of such treatment, together with or subsequently to adminstration of an antigen associated with the condition. The pathological condition may be a neoplastic disease, in which case the
35   antigen is derived from neoplastic cells alternatively the condition may be an infectious disease, and the antigen is derived from the infectious agent.

In these last two aspects of the invention it is particularly contemplated that the tumour antigen is a cancer-associated peptide such as Melan A, tyrosinase, GP100, or an antigen of the MAGE family, or a member of the

5   family of cancer antigens, such as ESO-I or a cancer testis antigen detectable by the SEREX method. The antigen may alternatively be derived from cancer cells or may be autologous, irradiated tumour cells, optionally in conjunction with GM-CSF.

10   Another important aspect of the invention provides a method of using a Type I interferon as an adjuvant for peptide-based anti-tumour vaccination strategies. In one embodiment of this aspect, a Type I interferon is administered locally or systemically at the

15   same time as, or 1 to 3 days after vaccination of a patient in need of such treatment with a tumour peptide or tumour cell lysate, optionally in conjunction with GM-CSF. In a second embodiment, this aspect of the invention provides a method using a type I interferon as an adjuvant for

20   vaccination against an infectious agent. Preferably the infectious agent is a bacterium, a virus, a yeast or a parasite.

It will be clearly understood that while the invention is defined above with reference to treatment of

25   human patients, it is also applicable to non-human mammals and therefore to veterinary treatment, including but not limited to treatment of domestic, companion or zoo animals.

Brief Description of the Figures

30   Figure 1 shows a comparison of antigen expression and morphology of cultured cells after 14 and 28 days of culture,

A)   Comparison of CD1a, CD83 and CD86 expression within the large-sized (high forward scatter)

35   population at day 14 (——) and day 28 (━━). DC cultures were grown in GM-CSF (10 ng/mL), TNF-α (20 ng/mL) and IL-4 (500 U/mL), and flow cytometry was performed at the

indicated times to assess antigen expression. One
representative experiment is shown. x-axis, fluorescence
intensity (logarithmic scale); y-axis, cell number.

B)   Cytospin preparations of unseparated,
cultured cells at day 14 (I) and day 27 (II). Staining was
performed by May Grunwald-Giemsa stain. Original
magnification for both panels: x200.

Figure 2 shows the immunophenotype of DC after 28
days in serum-free culture.

Analysis of DC by flow cytometry at day 28.
Culture conditions as in Figure 1A. DC (23% of total
cells) were gated on the basis of high forward and side
scatter properties as shown in A). In B), staining of this
cell population with the indicated antibodies (filled
histograms) is shown. The unfilled histograms represent
staining with the control antibody.

Figure 3 shows the assessment of antigen-
presenting function of mature DC.

A)   MLR. Stimulation index of DC cultures and
autologous PBL in the allogeneic MLR. The points represent
mean±SEM. DC cultures were grown as described in Figure 1A
for 26-28 days before inclusion in the assay (n=27). PBMC
controls were obtained by thawing CD34⁻ cells, originally
isolated and frozen at the time of CD34⁺ cell separation
(300-10,000 stimulators, 100,000 responders) (n=3).
Thymidine incorporation after 5 days was measured and
divided by the Thymidine uptake of responder cells alone to
calculate the stimulation index.

B)   MLPC. Comparison of the ability of sorted
DC and autologous PBMC to generate autologous, peptide-
specific CTL (n=3). DC cultures were grown as described in
Figure 1A. At day 27 of culture, DC were sorted by FACS,
and DC and PBMC were pulsed with influenza matrix peptide
and co-cultured with 10^6 autologous PBMC in an MLPC.
Numbers of stimulators are shown in the figure. At day 21
of the MLPC, cells were tested for their ability to lyse
matrix peptide-labeled T2 cells in a ^{51}Cr release assay.

Background lysis against T2 cells without peptide was always < 20% of maximal release, and was subtracted from experimental lysis percentages to give specific lysis results as shown here.

5   Figure 4 shows the cytokine optimization within serum-free DC cultures.

      A)   Effect of GM-CSF concentration and IL-4 on the percentage of CD1a+ cells in culture at day 14. Cultures containing GM-CSF (50 ng/mL) alone (n=2); TNF-α

10 (20 ng/mL) and IL-4 (500 U/mL) without GM-CSF (n=1); GM-CSF (10, 50 and 200 ng/mL) together with TNF-α (20 ng/mL) and IL-4 (500 U/mL) are compared. Each data set represents a single subject. CD1a expression within cultures was assessed by flow cytometry at day 14.

15   B)   Effect of TNF-α concentration on the percentage of CD86+ cells in culture. Cultures containing TNF-α (2.5, 20, 100 ng/mL), GM-CSF (10 ng/mL) and IL-4 (500 U/mL) are compared (n=3). CD86 expression was assessed by flow cytometry at day 28.

20   C)   Effect of IL-4 on the proportion of CD86+ cells between days 21 and 28 of culture. Cultures containing GM-CSF (50 ng/mL), TNF-α (20 ng/mL) with or without IL-4 (500 U/mL) are compared. The results of eight individual experiments are shown (mean±SEM: with IL-4:

25 39±7%, without IL-4: 25±6%). CD86 expression was assessed by flow cytometry between days 21 and 28.

      Figure 5 shows the effect of human serum on development of CD14-, CD83+ DC at day 28. Results of one representative experiment are shown (CD14: n=4; CD83: n=2).

30   A)   Cultures contained GM-CSF (50 ng/mL) and TNF-α (20 ng/mL).

      B)   as in A) with 10% human serum from day 0; x-axis: fluorescence intensity (logarithmic scale); y-axis: FSC.

35   Figure 6 shows the cell yield kinetics and early differentiation in serum-free cultures.

A)   Time course of overall cell yields in serum-free conditions is shown. Results are represented relative to the number of CD34$^+$ cells present at the initiation of the cultures (10^5 cells in 100µl: ●, ■; $2x10^5$ cells in 100 µl: ▲,▼,◆; all derived from leukapheresis products). Cells were maintained in the following cytokines: GM-CSF (40ng/ml), IL-4 (500U/ml) and TNF-α (20ng/mL).

B)   Cell differentiation within the first three days. Up-regulation in CD33 expression by the CD34$^+$ cells is observed between the start of culture (day 0) and day 3. Culture conditions as in A. Flow cytometry was performed at the indicated times to assess antigen status. One representative experiment is shown (n=4).

Figure 7 shows the time course of antigen expression over days 3-17).

10^5 CD34$^+$ cells were cultured in 100 µl cultures in 8 replicates in 96-well plates, and one well was harvested every second day for FACS analysis. Culture conditions were as in Figure 1A. Vertical rows of dot plots represent antigen expression in one well at one time point. Total cell population of one representative experiment is shown (n=3, all derived from leukapheresis products). Vertical gates to distinguish positive populations were set according to control Ab (first horizontal lane). Horizontal gates were set to distinguish large and small cell populations.

Figure 8 shows the morphology of sorted CD1a$^+$ DC and CD1a$^-$ cells at day 14

A)   Early DC stages showed temporary plastic adherence. Phenotype of CD11b^{bright} DC at day 14, 24 hours after sorting. Original magnification (OM) x180.

B)   I: Electron micrograph picture of sorted CD1a$^+$ dendritic cells (day 14). The bar indicates 1.5 µm,

II: Birbeck Granules in sorted CD1a$^+$ DC (day 14). The bar indicates 0.23 µm,

III: Mature DC (day 28) did not contain Birbeck Granules. The bar indicates 2.1 μm.

C)   Electron micrograph picture of sorted CD1a⁻ cells.

I: mononuclear phagocytic cell, containing phagocytic vacuoles. The bar indicates 1.2 μm.

II: granulocyte with segmented nucleus. The bar indicates 1.2 μm.

III: mononuclear, non-phagocytic cell. The bar indicates 1.8 μm.

Figure 9A shows the DC surface phenotype on days 14 and 28 by flow cytometry.

Phenotypic changes during DC maturation: CD34⁺ cells were cultured in GM-CSF, TNF-a and IL-4 and analyzed by flow cytometry after 14 days and 28 days. Cultures were gated to examine only the large cells (20% of total cells). Comparison between day 14 (——) and day 28 (——) is shown in a representative experiment. The position of the control cells labeled with non-specific antibody (IAG-11) is indicated by the horizontal line. x-axis: fluorescence height (log scale), y-axis: relative cell number.

Figure 9B shows CD11b expression in our representative culture at three different time points. Cultures and flow cytometry were performed as described for Figure 9A.

Figure 10 shows the differentiation of subpopulations sorted at day 13.

A)   Expression of CD11b on total cells after 13 days in serum-free culture. (Culture conditions as in Fig.1A). Three subpopulations were sorted according to FSC and CD11b expression: I=small, CD11b⁻ cells; II=small, CD11b⁺ cells; III=medium/large, CD11b^{bright} cells.

B)   Differentiation of subpopulation I (small, CD11b⁻) cultured until day 18 in GM-CSF, TNF-α and IL-4.

C)   Differentiation of subpopulation II (small, CD11b⁺) cultured until day 18 in GM-CSF, TNF-α and IL-4.

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D)   Differentiation of subpopulation III (medium/large, CD11b^{bright}) cultured until day 18 in GM-CSF, TNF-α and IL-4.

Figure 11 shows the allostimulatory capacity of
5 DC cultured in GM-CSF, TNF-α and IL-4 for days 14 and 28 using mixed leukocyte reaction

The allostimulatory capacity of 14 individual unsorted DC cultures was directly compared after 14-15 days (■) and 28 days (▼) using the same allogeneic responder
10 cells for both time points. DC were cultured in GM-CSF, TNF-α and IL-4, irradiated and co-cultured with 10^5 PBMC in triplicate wells. ^{3}H-thymidine uptake was measured after 5 days. Thymidine incorporation of responder cells alone was 1600±350 cpm. Results are shown as the mean±SE of 14
15 individual experiments (* $p<0.05$, ** $p<0.01$).

Figure 12 shows the superior effectiveness of mature Dendritic Cells expressing low levels of CD11b in stimulating CD8+ peptide-specific lymphocytes, compared to immature DC expressing high amounts of CD11b.

20   Figure 13 shows the effect of addition of human serum to cultures containing GM-CSF, TNF-α and IL-4 on the expression of CD1a and CD86. Cultures were split 3 days before analysis and human serum was added into one half culture.

25   A)   Normal development of serum-free cultures: accumulation of a population of large sized, CD1a+ DC which did not express CD86.

B)   Addition of human serum 3 days previous to analysis by flow cytometry. Similar expression of CD1a and
30 CD86 in culture suggesting the quick maturation of CD1a+ DC in response to activating factors in human serum.

Figure 14 shows the screening for cytokines for activating effects on DC.

CD34$^+$ cells were cultured in 96 well plates in
35 GM-CSF, TNF-α and IL-4 (GTI). At day 14, cytokines were added at the concentrations shown in Table 3. Cultures were analyzed by flow cytometry at day 17. The proportion

of activated DC (HLA-DR^{+++} and/or CD86$^+$) was calculated referring to the mean expression in multiple control cultures (GTI) as 100%. SDs were calculated for all protocols and a no-difference interval of two standard

5   deviations above control levels of expression was chosen as a cut-off level for increased expression (shaded area). Only human serum and IFN-α2a were capable of inducing an increase >2SD in the proportion of activated DC in culture.

Figure 15 shows the concentration dependent

10   effect of type I IFNs on DC maturation.

Direct comparison of titrations of IFN-α2a (■) (n=3), IFN-α8 (●) (n=1) and IFN-β (▼) (n=1), on antigen expression of DC. DC cultured from a single donor were grown in GM-CSF, TNF-α and IL-4 until day 14, type I IFNs

15   were added daily between day 14 and day 17. FACS analysis was performed on day 17. Cell populations were gated to include only the large cells (20% of total cells).

Figure 16 shows the induction of CD83 expression in DC cultures by IFN-α2a.

20   CD83 expression of DC cultures without (A) or with (B) IFN-α2a added for 3 days. Progenitor cells were cultured under standard serum-free conditions. On day 14 the culture was split and IFN-α2a (1000 U/ml) was added into one half. FACS analysis was performed on day 17.

25   Vertical gate indicates fluorescence of 98% of cells with control antibody.

Figures 17A and 17B show that the effect of IFN-α on DC between day 13-16 depended on the previous exposure of these cultures to IL-4 (n=3). (GT: GM-CSF (50

30   ng/mL)+TNF-α (20 ng/mL). GTI: GM-CSF, TNF-α+IL-4 (500 U/mL)).

A)   CD80 expression in cultures assessed by flow cytometry on day 16.

B)   CD86 expression in cultures assessed by

35   flow cytometry on day 16.

Figure 18 compares the percentage of activated DC in paired cultures with and without 3 days' exposure to IFN-α.

Figure 19 shows the allostimulatory capacity of DC activated by IFN-α in the same six cultures as in Figure 18.

A) The effect of IFN-a2a on the allostimulatory capacity of unsorted cultures of DC was studied after 17 days culture in GM-CSF, TNF-a and IL-4 (■) compared to paired cultures with addition of IFN-a2a (1000 U/ml) days 14-17 (♦), (n=6), p<0.05.

B)   Allostimulatory function of DC sorted on day 18. Culture was split on day 14 and either continued in GM-CSF, TNF-α and IL-4 (■) or in these three cytokines together with IFN-α2a (1000 U/ml) added on days 14-18 (♦). On day 18, large DC were sorted and the MLR was performed as described in the Materials and Methods.

Figure 20 shows the effect of IFN-a on migration and activation of skin-derived dendritic cells.

A)   Cumulative number of migratory skin-derived DC. One representative experiment is shown (n=6). Split skin was cut into paired samples and cultured in serum-free medium with or without IFN-α2a (3000 U/ml). DC migrating out of the skin were counted every 12 hours.

B)   CD80 expression of migratory LC after 48 hours: (i) no IFN-α, (ii) with IFN-α. X-axis: fluorescence-height, Y-axis: forward scatter. Gate defines fluorescence with a control antibody.

Detailed Description of the Invention

The invention will now be described in detail by way of reference only to the figures and to the following examples.

Materials and Methods
Media

The serum-free medium X-Vivo 20 was purchased from BioWhittaker, Walkersville MD. Cell lines were grown
5   in RPMI 1640 (Trace Biosciences, Melbourne, Australia) supplemented with 20 mM HEPES, 60 mg/l penicillin G, 12.6 mg/l streptomycin, 2 mM L-glutamine, 1% non-essential amino acids and 10% heat-inactivated FCS (CSL, Melbourne, Australia).

10

Recombinant Human Cytokines, Antibodies, Peptides

The following cytokines were added to DC cultures: TNF-α (2.5-100 ng/ml) (R&D Systems, Minneapolis, MN); GM-SCF (10-200 ng/ml) (Schering-Plough, Sydney,
15   Australia), SCF (100 ng/ml) (AMGEN, Thousand Oaks, CA). IL-4 (100-1000 U/ml) (Schering-Plough New Jersey, NJ). The following commercial monoclonal antibodies (mAb) were purchased: FITC-conjugated IgG1 isotype control; OKT6, anti-CD1a; PE-conjugated T4, anti-CD4; B3, anti-CD22;
20   IL-2RI, anti-CD25; MY9, anti-CD33; NKH1, anti-CD56; AICD 58, anti-CD58; (Coulter Corp., FL); PE-conjugated IgG1 isotype control; HPCA-2, anti-CD34; (Becton Dickinson, San José, CA); LFA-1, anti-CD11a/18; Kb90, anti-CD11c; PE-conjugated TUK4, anti-CD14; (Dako
25   Corp. Carpenteria, CA); AT 10, anti-CD32/FcyRIII; BB-1, anti-CD80/B7/BB1; (Serotec, Oxford, UK); 5C3, anti-CD40; IT2.2, anti-CD86/B70/B7-2; (PharmIngen, San Diego, CA); FITC-conjugated sheep anti-mouse mAb; Leu11b, anti-CD16; (Silenus, Miami, FL). The following mouse Ab were kindly
30   provided by Dr A. Boyd, The Walter and Eliza Hall Institute, Melbourne: IAG-11 negative control, W6/32, anti-HLA-A,B,C; Ia, anti-HLA-DR; LYM-1, anti-CD2; T3, anti-CD3; T4, anti-CD4; T8, anti-CD8; OKM-1, anti-CD11b; FMC 17, anti-CD14; WMG1, anti-CD15; FMC 63, anti-CD19; B1,
35   anti-CD20, UCHL1, anti-CD45RO; FMC 71, anti-CD45RA; ICAM-1, anti-CD54. The influenza matrix peptide (residues 57-68:

GILGFVFTL) was a gift from Dr. P. Romero, Ludwig Institute
for Cancer Research, Lausanne, Switzerland.

Cell Sources, Cell Line

Human bone marrow (BM) and leukapheresis harvest
samples were obtained from normal donors and from patients
of the Department of Medical Oncology and Clinical
Haematology, Royal Melbourne Hospital, Melbourne. Patients
with non-Hodgkin's lymphoma or solid tumours received stem
cell-mobilizing chemotherapy and granulocyte colony-
stimulating factor (G-CSF) as part of their treatment. Rib
segments removed during thoracotomy from patients with lung
cancer were obtained from the Department of Surgery, Austin
Hospital, Melbourne. The human T2 cell line (Hosken and
Bevan, 1990) was kindly provided by Dr. P. Romero, Ludwig
Institute, Lausanne, Switzerland. Human K562 cells (Lozzio
and Lozzio, 1975) were a gift from H. Zogos, Rotary Bone
Marrow Research Institute, Melbourne.

Cell Separation and DC Cultures

Mononuclear cells were prepared by density
centrifugation on Ficoll-Paque (Pharmacia Biotech, Uppsala,
Sweden). Using 14 gauge needles, rib segments were flushed
with RPMI to mobilize BM cells. Residual red cells were
lysed with red cell lysis buffer (7.79 g/L NH_4Cl, 0.037 g/L
EDTA, and 1 g/L $NaHCO_3$) and $CD34^+$ cells were separated with
the MACS CD34 progenitor cell isolation kit (Miltenyi
Biotec, Sunnyvale, CA) (Lansdorp, 1989). Purity of the
$CD34^+$ fraction was assessed by flow cytometry using HPCA-2
mAb (Lansdorp, 1989) and was consistently over 96%. Cells
(10^6 ml-4 x 10^6 ml) were cultured in 100 µl X-Vivo 20 with
cytokines in 96 well microcultures (Nunc). 50-100% fresh
medium and cytokines were added every 2 to 3 days.
Proliferating confluent cultures were transferred with an
Eppendorf pipette into progressively larger tissue culture
plates (48 well plates (Falcon), 24 well plates (Nunc),
12 well plates (Flow Lab. VA) and 6 well plates (Nunc)).

The CD34-fraction was used for HLA typing (Victorian Tissue
Typing Service, Royal Melbourne Hospital). The remaining
cells were frozen in FCS with 10% DMSO (Merck, Melbourne,
Australia) for use as autologous responders or stimulators
5   in later functional assays.

Morphology, Flow Cytometry and FACS
        Cytocentrifuge preparations were prepared by
applying 10^4 cells to glass slides and spinning for 10 min
10   at 300 rpm (Shandon, Cytospin 2). These were air-dried and
stained with May-Grunwald/Giemsa stain.
        Cells for flow cytometry were incubated with the
primary mAb in blocking buffer (Phosphate buffered saline
(PBS) (2.275 g/L Na_2HPO_4 (anhydrous), 0.552 g/L $Na_2HPO_4xH_2O$,
15   7.0 g/L NaCl in deionized distilled water) with 10% human
AB-serum) for 5 min at room temperature (RT), washed in
PBS/0.01% Na azide and, where the primary mAb was not
directly FITC-or PE-conjugated, incubated with the
FITC-labeled secondary mAb. Cells were fixed in PBS/2%
20   Formaldehyde/0.01% Na azide/1% BSA. The immunophenotype
was determined using a FACScan flow cytometer (Becton
Dickinson). FACS was performed on either a FACStar plus or
a modified FACS II flow cytometer.

25   *Mixed Leucocyte Reaction (MLR)*
        Peripheral blood mononuclear cells (PBMC) of
healthy laboratory volunteers (10^5 per 100 µl culture) were
used as responders. Cells were cultured for 5 days in RPMI
with 10% FCS in 96 well round-bottomed plates. Stimulators
30   were either DC culture populations or frozen and thawed
CD34⁻ cells (60-80% viability) from the same patient.
After irradiation (3000 rad), stimulator cells were plated
out in triplicate over a range of concentrations
(10,000-3,000-1,000-300 per well). After 5 days, cells
35   were incubated with 0.5 µCi/well ³H-thymidine (DuPont,
Sydney, MA) for 20 hours, transferred on to a glass fibre
filter (Wallac, Turku, Finland), and ³H-thymidine-

incorporation into DNA was measured using an
LKB 1205 Betaplate scintillation counter (Wallac).

Mixed Leukocyte Peptide Cultures (MLPC)

5   The capacity of DC and DC sub-populations to
stimulate peptide-specific CD8^{+} CTL was assessed. DC or
autologous PBMC (CD34^{-} fraction) of HLA-A2^{+} patients were
pulsed with influenza matrix peptide (10 µg/ml) in the
presence of β_2-microglobulin (2.5 µg/ml, Sigma Aldrich,
10   NSW) in X-Vivo 20 for 45 min at 37°C. Stimulators were
washed and co-incubated with 10^6 autologous PBMC
(CD34[-]fraction) as responders in X-Vivo 20, 10% pooled
human A serum and 10-20 U/ml IL-2 (Pepro Tech, Rocky, NJ).
To improve cell-cell interactions during the first week,
15   cultures were established in hanging drops[11] in Terasaki
plates (Nunc, 20 µl drops containing approx. 10^5 cells per
drop). After 7 days, cultures were transferred into
96 well flat-bottomed plates and re-stimulated with
peptide-pulsed, irradiated, autologous PBMC at day 7 and
20   14. After 3 to 4 weeks, cultures were examined for
CD8^{+} lymphocytes by staining with anti-CD8 mAb, and flow
cytometry and ^{51}Cr release assays were performed, depending
upon growth and expansion of responder cells.

25   *^{51}Cr Release Assay*
          When appropriate, responder cells from MLPC were
assessed for peptide-specific lysis. T2 cells were labeled
with 100 µCi of Na(^{51}Cr)O$_4$ (Du Pont NEN) with 10 µl of W6/32
ascites for 2 hours at 37°C on a rotating stand. After
30   3 washes in RPMI, cells were incubated with 2.5 µg/ml
β_2-microglobulin ± 1 µg/ml peptide in X-Vivo 20 for 1 hour
at 37°C, washed once, and re-suspended in RPMI with 10% FCS
at 10^4 cells/ml. Responder cells were pre-incubated at
different concentrations (from 10^3 to 3×10^4 cells/ml) and
35   with 5×10^4 K562 for at least 30 min. K562 is a Natural
Killer cell (NK)-target and was added to inhibit non-
specific lysis by contaminating NK cells. 1,000^{51}Cr-

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labeled target cells± peptide were added for 4 hours.
Control wells containing K562 and labeled target cells ±
peptide in medium alone or with 1% Triton-X were used to
assess spontaneous release (SR) and maximal release (MR)
5 respectively. Plates were centrifuged (300 rpm, 5 mins)
and 75 µl of supernatant was loaded on to a Lumaplate TM
containing solid scintillant (Canberra Packard, IL) and
air-dried. To calculate peptide-specific lysis, background
lysis in wells with unpulsed targets was substracted from
10 lysis of peptide-pulsed targets. Thus, the percentage of
peptide-specific lysis at each E:T ratio was calculated as
follows:

= % lysis with peptide pulsed targets (pepT)-% lysis with unpulsed targets
15 (T)

$$= \frac{\text{experimental release (pepT)-SR(pepT)}}{\text{MR(pepT)-SR(pepT)}} - \frac{\text{experimental release(T)- SR(T)}}{\text{MR(T)-SR(T)}}$$

Cytokine deprivation and phagocytosis
20   Bulk DC cultures and sorted DC were washed, then
cultured in X-Vivo 20 with 10% human serum for 5 days in
order to assess the stability of their phenotype in the
absence of exogenous cytokines and their ability to
phagocytic *Candida albicans* cells. As a control, adherent
25 PBMC were obtained by density centrifugation as described
above from blood samples of normal volunteers and incubated
for 1 hour at 37°C in X-Vivo 20 with 20% human serum (HS).
Non-adherent cells were removed, and remaining adherent
cells incubated in the same medium for another 5 days. An
30 assay to examine the phagocytic ability of DC was performed
using heat-killed *Candida albicans*. Cells were washed,
cultured in X-Vivo 20 with 10% HS, and *C. albicans* was
added for 15 mins at 37°C, 10% CO_2. Phagocytosis was
examined in untreated (opsonizing) and heat-inactivated
35 (non-opsonizing) HS over 15 mins, 1 and 2 hours incubation.
Cytospins were performed and examined by light microscopy.

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<u>Example 1</u>   <u>Characterization of Mature DC Derived from</u>
                    <u>Human CD34⁺ progenitor Cells Under Serum-</u>
                    <u>Free Conditions</u>

Phenotypic and Morphological Changes of DC in Serum-Free
5   *Cultures.*

Purified $CD34^+$ progenitor cells were cultured in
serum-free medium (X-Vivo 20) in the presence of GM-CSF
(10-200 ng/ml) and TNF-α (2.5-100 ng/ml), with and without
IL-4 (100-1,000 U/ml). In the absence of serum, cell
10 growth was density-dependent. Since cells failed to grow
when seeded at densities lower than 5×10^5 cells/ml (n=4),
$CD34^+$ cells were seeded at $>10^6$ cells/ml.

DC development in serum-free conditions was
observed as a process of phenotypic and morphological
15 evolution which took 3 to 4 weeks. Figure 1 shows a brief
summary of the phenotypic and morphological events which
occur during days 14 and 28. At day 14, a proportion
(10-25% of bulk culture cells) expresses CD1a, a MHC-like
surface antigen known to be expressed on epidermal
20 Langerhans cells. However, DC-associated markers like CD83
and CD86 are only expressed on a minority of the cells and
gradually increase until day 28. This process is referred
to herein as DC *maturation*. This is illustrated in
Figure 1A, which shows the up-regulation of CD83 and CD86
25 and down-regulation of CD1a on a subpopulation of large
cells between days 14 and 28. Figure 1B depicts the
corresponding morphological changes. Panel I shows cells
at day 14 which included a small percentage of DC which
adhered to plastic surfaces (arrows) and were detached here
30 by pipetting. At day 28, most DC were non-adherent and
displayed a round phenotype with fine dendrites, as shown
in panel II.

Phenotype of Mature DC at day 28
35   The surface antigen expression pattern of these
mature large cells is shown in Figure 2. The cells showed
high expression of HLA-A,B,C, HLA-DR, CD40, CD54, CD58 and

CD86, and were positive for CD1a, CD4, CD11a, CD11b, CD11c, CD80, CD83 and CMRF44. They did not express T and B cell markers such as CD2, CD3, CD5, CD8, CD19 and CD20, Fc receptors such as CD16 and CD32, nor monocyte-associated markers such as CD14 and CD45RA. Thus all antigens associated with mature DC (CD80, CD83, CD86, CMRF44) as well as adhesion molecules (CD11a, CD11c, CD54, CD58) are expressed, whereas markers of other lineages are lacking.

Morphologically, they displayed the typical DC features (Mackensen *et al*, 1995) of high forward and side scatter by flow cytometry, a corona of fine dendrites and indented nuclei. As well as the DC, the cultures contained 20 to 80% small cells (Figure 2A). These were negative for CD1a, CD4 and CD86, whereas a subpopulation expressed CD11b and CD15.

Example 2   Function of DC as Antigen-presenting Cells

To evaluate their allostimulatory capacity, DC were tested in MLR and MLPC assays. As shown in Figure 3A, cultures containing DC were far more potent stimulators of allogeneic lymphocytes than PBMC from the same patient (n=10). In order to compare the ability of DC and PBMC to present peptide antigens to CD8$^+$ CTL, MLPC assays were performed using an HLA-A2-restricted peptide (GILGFVFTL) derived from the influenza matrix protein (Figure 3B). As few as 2,800 sorted CD4$^+$ DC were sufficient to stimulate specific cytotoxicity against peptide-pulsed T2 target cells. In contrast, at least 10^5 peptide-pulsed PBMC were required to obtain a measurable effect (n=3). In these experiments, DC were sorted according to cell size to avoid blocking of functional receptors by antibodies (CD1a, CD80, CD86). In order to confirm that peptide-specific lysis was due to CD8$^+$ CTL, effector cells of one MLPC were sorted into CD8$^+$ and CD8$^-$ cells. Only the CD8$^+$ fraction had the ability to lyse peptide-pulsed target cells.

Thus these DC are characterized functionally as non-phagocytic, potent antigen-presenting cells (APC),

stimulating allogeneic lymphocytes in the MLR, and
autologous peptide-specific cytotoxic T-lymphocytes in the
MLPC.

5   Example 3   Cytokine deprivation and phagocytosis
             The ability of DC to maintain their phenotype
when removed from the exogenous cytokine culture
environment was tested by culturing the cells in X-Vivo 20
with 10% HS for 5 days. In contrast to adherent PBMC
10   (n=2), mature DC did not transform into adherent
macrophages. After 5 days in human serum, DC maintained
CD83 expression and did not express monocyte/macrophage
associated markers such as CD14 and CD32.
             Assessment of their phagocytotic ability showed
15   that DC at days 21 to 28 of culture were able to bind but
not phagocytose C. albicans (75 cells counted, n=5
experiments after 15 and 40 mins in opsonizing medium, n=2
experiments after 1 h, 2 h, and 5 days in non-opsonizing
medium). In contrast, adherent PBMC (2.9 ± 0.3 Candida per
20   cell (n=76 cells counted) and the cells of the small, non-
dendritic fraction that were also present in DC cultures
(4.5 ± 0.5 Candida per cell, n=92 cells counted) readily
phagocytosed the Candida after 40 mins in opsonizing
medium.
25

     Example 4   Characterization and Optimization of Serum-
                      Free Culture Conditions
     Titration of Cytokines
             In the absence of serum, cell growth was density-
30   dependent. Since cells failed to grow when seeded at
densities lower than 5×10^5 cells/mL (n=4), CD34+ cells were
seeded at $1-3 \times 10^6$ cells/mL.
             To determine conditions for optimal DC generation
in serum-free cultures, GM-CSF, TNF-α and/or IL-4 were
35   titrated over a range of concentrations. DC development
was quantified using the expression of CD1a at day 14 and
of CD86 at day 28.

Day 14   Although not sufficient to stimulate DC growth in the absence of other growth factors, GM-CSF was absolutely required for DC growth (Figure 4A). No significant differences in proportion or yield of CD1a^{+} cells were observed in a concentration range between 10 and 200 ng/ml GM-CSF. In the presence of GM-CSF (50 ng/ml), TNF-α promoted DC development into CD1a^{+} cells (Figure 4A). 20 ng/ml TNF-α gave maximum stimulation of CD1a expression, and this concentration was chosen for all further experiments. DC growth was optimal when TNF-α was present for the entire culture period (14 ± 2% CD1a^{+} cells at day 14, n=5). Addition of TNF-α from day 0 to 5 into cultures containing both GM-CSF and IL-4 resulted in 8 ± 1% (n=4) and from day 10 to 14 in 4 ± 0.7% (n=2) CD1a^{+} DC. IL-4 further promoted the growth of CD1a^{+} cells (Figure 4B). At day 14, higher percentages of CD1a^{+} cells were observed in the presence of IL-4 (no IL-4: 4.8 ± 1%, with IL-4: 14.2 ± 1%, n=5).

Day 28   The highest percentages of CD86^{+} DC were observed in cultures containing higher concentrations of TNF-α (20-100 ng/ml) in combination with IL-4 (500 U/ml) (Figure 4B). As observed with CD1a expression at day 14, the combined action of all three cytokines (GM-CSF, TNF-α and IL-4) was required for optimal expression of CD86 on DC at day 28 (Figure 4C).

Example 5   <u>Differentiation of CD14^{+} Macrophages in the</u>
                 <u>Absence of IL-4 and in the Presence of</u>
                 <u>Human Serum From Day 0</u>

        If CD34^{+} cells were cultured in the absence of IL-4, no CD14^{-}, CD11b^{+++}, CD1a^{+} cells developed in significant numbers during the first 2 weeks (not shown).

        At day 14 to 16, in the absence of IL-4, cultures contained 26 ± 2.6% (n=16) of medium-sized, CD14^{+} cells, in addition to a smaller population of large CD14^{-} cells (5% ± 1%, n=16). After day 14, the CD14^{+} population gradually reduced, even without adding IL-4 to the cultures

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(11.7% ± 3.3% at day 28, n=9). In contrast, the large
CD14$^-$ population expanded to 32.3% ± 13.8% of the culture
at day 28, n=9 (Figure 5A). The addition of IL-4 from
day 0 gave rise to CD14$^-$ DC, even if IL-4 was removed from
5   cultures as early as day 7 (n=3). Late addition (day 10 to
16) of IL-4 to cultures containing CD14$^+$ cells (n=5) or to
sorted CD14$^+$ cells (n=2) also decreased CD14 expression
(1.4 ± 0.5% CD14$^+$ cells at day 16, n=5).

        Interestingly, in the absence of IL-4, a
10 spontaneous reduction of CD14 expression combined with
acquisition of a mature DC phenotype was observed between
days 14 and 28. This effect did not occur if HS was
present from the start in these cultures. We hypothesize
that IL-4 is produced in serum-free cultures, leading to
15 the development of mature DC from CD14$^+$ precursors. HS
seems to favour the differentiation into CD14$^+$ monocytes/
macrophages if present during the whole culture period.
However, mature DC were not inhibited or reverted by HS.

        This automaturation of cultures containing CD14$^+$
20 cells in the absence of IL-4 at day 14 into CD14$^-$, CD83$^+$
DC-containing cultures at day 28 was completely abolished
if HS was present from day 0. In the presence of HS all
cells expressed CD14 and a monocyte/macrophage phenotype,
as shown in Figure 5B. This indicates that HS favours
25 monocyte development, and that serum deprivation may be
crucial for DC development *in vitro*.

Example 6   DC Yields in Serum-free Conditions and
                   Effects of Stem Cell Factor (SCF)
30   The variation in total cell yields with time
under serum-free conditions is shown in Figure 6A.
Regardless of TNF-α concentration, an early drop in cell
numbers followed by a gradual rise was observed. In
parallel with this drop in yield there was an increase in
35 the expression of CD33 in the first 3 to 5 days of culture
as shown in Figure 6B, indicating that the cultures were

not able to support the survival of non-myeloid cells
(n=6).

Different sources of CD34$^+$ cells were compared.
These included leukapheresis harvests, BM aspirates and rib
5   fragments from normal donors as well as from cancer
patients. The yield of CD34$^+$ cells/ml leukapheresis
product was recorded for 19 samples (8 patients). Yields
ranged from 2 x 10^5 to 5 x 10^7, with a median of 1.9 x 10^6
CD34$^+$ cells per ml leukapheresis product. Table 1 shows
10   overall cell yields and the proportion of CD1a$^+$DC obtained
at day 16 from 12 leukapheresis harvests, 4 rib segments,
and 3 normal BM samples, all performed during one 3 month
period. In 4 cases, total cell numbers did not exceed the
number of seeded CD34$^+$ cells. These results were
15   unpredictable, and the numbers are too small to relate
yields to cell source or to the patient's underlying
disease (Non-Hodgkin's lymphoma (n=1), Hodgkin's disease
(n=1), myelodysplasia/acute myeloid leukaemia (n=1), lung
cancer (n=1). Overall, an average of 2.8-fold expansion of
20   total cell numbers was observed. Of these, DC constituted
about 20% of cells in the cultures at day 16.

An attempt was made to increase DC yields by
including SCF. Addition of SCF (100 ng/ml) from day 0
doubled total cell yields (Table 2) without altering the
25   percentage and phenotype of DC. FCS and human serum (10%
from day 0) were likewise able to increase yields
(Table 2).

Table 1
Cell Yields After 16 Days of Culture[1]

Sample	Cell number ($\times 10^{-6}$)	% CD1a$^+$ Cells
Lh^a	1.9	17
Lh^a	2.8	21
Lh^a	1.9	27
LH^a	2.6	20
LH^a	5.5	21
LH^a	5.6	13
LH^a	5.2	20
LH^a	0.8	nd
LH^b	0.7	23
LH^c	3.5	21
LH^d	0.8	nd
LH^e	4.3	14
LH^e	1.9	13
LH^e	3.3	nd
n=14	2.9 + 0.5	19 + 1.3
BM^{rib}	2.6	27
BM^{rib}	3.0	19
BM^{rib}	2.2	22
BM^{rib}	4.4	29
BM^{rib}	4.4	22
BM^{rib}	2.0	22
BM^{rib}	1.3	27
BM^{rib}	3.0	20
BM^{rib}	0.12	24
n=9	2.5 + 0.4	23.5 + 1.1
nBM	5.2	10
nBM	1.9	29
nBM	5.0	26
n=3	4 + 1.1	22 + 5.9

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1   CD34$^+$ HPC were cultured for 16 days in the
presence of GM-CSF (10 ng/mL), TNF-α (20 ng/mL) and IL-4
(500 U/mL), at which time cells yields were assessed and
CD1a expression evaluated.   Yields are calculated based
5   upon a starting inoculum of 10^6 CD34$^+$ HPC.   LH
(leukapheresis harvest), BM (bone marrow), nBM (normal bone
marrow);

a   Non Hodgkin's Lymphoma (n=8);

b   Hodgkin's Disease (n=1);

10   c   Acute Myeloid Leukaemia (AML) (n=1);

d   Myelodysplasia-AML (n=1);

e   Breast Cancer (n=3)

rib   Lung Cancer (bone marrow of rib fragments)

15

Table 2
Expansion of Cell Yields[1]

Culture condition	n	Yield (%)
GTI	11	100
GTI+SCF	6	230 ± 33
GTI+FCS (10%)	3	221 ± 73
GTI+HS (10%)	2	620 ± 92

20

1   CD34$^+$ HPC were cultured in the presence of GM-CSF
(10 ng/mL), TNF-α (20 ng/mL) and IL-4 (500 U/mL) (GTI),
with the addition of either SCF (GTI+SCF), Foetal Calf
Serum (GTI+FCS) or Human Serum (GTI+HS), for 16 days, at
25   which time cells yields were assessed.   Yields are shown as
mean±SE relative to cultures without SCF/serum (=100%).

30

<u>Example 7</u>   <u>Time Course of Differentiation and</u>
              <u>Maturation of DC from Human CD34$^+$</u>
              <u>Progenitor Cells Under Serum-Free</u>
              <u>Conditions: Complex Phenotypic and</u>
5   <u>Functional Changes</u>

a) *DC arise from myeloid precursors*

       DC development in serum-free conditions was
observed as a process of phenotypic and morphological
evolution which took 3-4 weeks. The earliest steps of
10 differentiation coincided with the early phase of cell
death and resulted in a population of myeloid, CD32$^+$,
CD33$^+$, CD34$^+$ progenitor cells around day 3 (Figure 6).

b) *DC development between days 3-14*

15   The myeloid progenitors gradually gave rise to a
population of large, CD1a$^+$, CD14$^-$, CD32$^-$, CD40$^+$, HLA-DR$^+$
cells. No early peak of CD1a$^+$ precursors was observed
around day 6, and no expression of CD14 was found at any
time point in the culture (Figure 7). These large, CD1a$^+$
20 cells were further characterized phenotypically. At day
14, CD1a$^+$ cells expressed low levels of CD4 but no DC
activation markers such as CD80, CD83, CD86 or CMRF44 (not
shown). They were positive for CD4, CD11b, CD11c, CD13,
CD33, CD58 and CD54 (not shown). Between days 3-15,
25 cultures contained a significant proportion of loosely
adherent cells with dendritic morphology (Figure 8). It
was noted that depletion of non-adherent cells from these
cultures removed CD1a$^+$ cells (not shown). At day 14, 3
cultures were sorted into CD1a$^+$ and CD1a$^-$ cells. A
30 significant proportion of CD1a$^+$ DC showed Birbeck Granules
(BG) (20-40%) (Figure 8). BG were never seen in CD1a$^-$
cells. At day 14, the CD1a$^-$ cells were a heterogeneous
population including phagocytic cells and immature cells
(Figure 8C). Small cells were CD1a$^-$, CD14$^-$, CD40$^-$, CD32$^+$,
35 and expressed HLA-DR for a prolonged period (Figure 9A).
The expression of HLA-DR was gradually lost as these cells

matured.   The myeloid marker CD13 was likewise expressed on all small cells (not shown).

c)   *Phenotypic changes of maturing DC between day 14 and 28*

5   In serum-free cultures of human CD34$^+$ progenitor cells, two stages of DC differentiation were observed.   In the early phase of culture, (10-16 days), a population of large, adherent, Langerhans-like cells developed.   These cells were CD1a^{+++}, CD11b^{+++}, CD14$^-$, CD80$^-$, CD83$^-$, CD86$^-$,

10 HLA-A,B,C$^+$ and HLA-DR$^+$ cells (Figure 9A; ———).   20-40% of CD1a$^+$ cells sorted on day 14 contained Birbeck Granules (see Figure 8).   During the next 2 weeks (late phase, days 17-28), this population spontaneously matured into non-adherent APC with up-regulation of HLA-A,B,C, HLA-DR,

15 costimulatory molecules (CD80, CD86) and DC lineage associated antigens (CD83, CMRF44).   Simultaneously, down regulation of CD11b and CD1a was seen (Figure 9A; ———). The loss of these antigens was slow and was complete between days 28-40.   No Birbeck Granules were found in

20 activated DC.   This late phase (d14-28), now referred to as phenotypic maturation, was associated with a loss of adherence to plastic (not shown).

d)   *Asynchronous maturation of intermediate DC between days*
25 *14-28*

            Heterogeneity of expression was found in antigens whose levels changed between days 14-28.   This raised the question whether the large sized population at this time point consisted of 2 distinct dendritic and non-dendritic

30 cell populations or whether asynchronous maturation of immature DC temporarily led to the co-existence of immature and mature DC. The experiments shown in Figures 9B and 10 support the second explanation.   Figure 9B shows the CD11b expression in one representative culture at three different

35 time points.   Most large sized cells expressed high levels of CD11b at day 16.   At day 28, 50% of the large cells had down regulated CD11b to low levels, whereas 50% still

expressed high levels of this antigen (cells were gated for size as indicated by the horizontal line and for antigen expression by the dotted line). However, at day 37, more than 80% of the large cells were CD11b^{low}. In parallel, a
5   similar percentage of large cells expressed CD86 (not shown). This suggested that the mature phenotype was acquired asynchronously and was still in process at day 28. To confirm this hypothesis and to confirm the assumption that only the large sized population was capable of
10 differentiating into mature DC, cells from these cultures were sorted at day 13 on the basis of CD11b expression and cultured until day 18. Figure 10A shows three different populations sorted at day 13: I=small, CD11b$^-$ cells, II=small CD11b$^+$ cells, III=medium/large CD11b^{bright} cells.
15 Only the CD11b^{bright} cells expressed CD1a (not shown). When cultured for 5 days in the presence of GM-CSF, TNF-α and IL-4 until day 18, 41% of the small CD11b$^-$ population (I) had developed into small, CD11b$^+$ cells by d18 and 19% of the small CD11b$^+$ cells (II) developed into large, CD11b^{bright}
20 cells (Figure 10B). This confirmed a) that DC differentiated asynchronously from progenitor cells, and b) that a proportion of the small cells still represented immature precursors at least until day 14. In contrast, all of the large, CD11b^{bright} population (III) differentiated
25 into non-adherent cells with the phenotype of mature DC (CD14$^-$, CD86$^+$, CMRF-44$^+$, CD11b$^{-/low}$), (Figure 10B).
        The small cell population remaining after 28 days was HLA-DR$^-$. 50% of these were granulocytes on the basis of CD15 expression.
30

e) *Functional maturation of DC between day 14 and 28*
        The antigen presenting capacity of cells from unsorted cultures was examined on days 14 and 28. The results are shown in Figures 11 and 12. Direct comparison
35 of cells from both time points was possible because the same donor of responder cells was used within the paired experiments. Figure 11 shows the significantly increased

allostimulatory capacity of day 28 DC cultures compared to day 14 DC cultures. Note that the assays were performed with unsorted cultures containing only 20% DC.

Sorted CD11b^{low} and CD11b^{bright}/CD4$^+$ DC were
5   compared in the MLPC. Figure 12 shows that mature CD11b^{low} DC are superior to immature CD11b^{bright} DC in stimulating CD8$^+$, peptide-specific CTL.

Our results show that CD34$^+$ progenitor cells differentiate in our serum-free system from myeloid
10   progenitors to immature DC and finally into mature DC. The presence of Birbeck Granules and the typical phenotypic changes during DC activation suggest that our culture system favors the development of Langerhans-like DC, similar to epidermal Langerhans cells.

15

Type I Interferons Enhance the Terminal Activation of Dendritic Cells

    Example 8   Serum Factors in Normal Human Serum Enhance
20   CD86 Expression Within 3 Days
         The gap between the expression of CD1a (immature DC) and CD86 (mature DC) in the serum-free cultures (Figure 13A) could be closed by the addition of human serum 3 days prior to FACS analysis, as shown in Figure 13B.
25   This led to a search for cytokines which could be involved in this process.

    Example 9   Cytokine Effects on the Late Phase of DC
                       Differentiation
30   To determine whether addition of exogenous cytokines could accelerate DC maturation and to identify those endogenous factors which might be acting, we screened a variety of cytokines for their effects on DC phenotype in serum-free cultures. HLA-DR and/or CD86 up-regulation were
35   used as markers of maturation. For this assay, cytokines were used at a concentration previously found to be active in other systems, as summarised in Table 3.

Table 3

Factors Screened for Activating Activity in DC Culture

Factor tested	Concentration	Source
Interleukin (IL)-1α	10 ng/ml	Hoffmann LaRoche, N.J., gift of Dr. I. Campbell, Dept. of Medicine, Royal Melbourne Hospital (RMH), Melbourne, Australia
IL-1β	100 pg/ml	R&D systems, Minneapolis, MN
IL-2	100 U/ml	PeproTech, New Jersey, NJ
IL-3	100 ng/ml	Dr. Glenn Begley, The Walter and Eliza Hall Institute (WEHI), Melbourne, Australia
IL-4	1000 U/ml	Schering-Plough, Kenilworth, NJ
IL-6	20 ng/ml	Dr. R. Simpson, Ludwig Institute for Cancer $Research (LICR), Melbourne, Australia
IL-7	20 ng/ml	Serotec, Oxford, UK
IL-8	100 ng/ml	Genentech Inc., CA, gift of Dr. I. Campbell, RMH
IL-10	100 U/ml	Schering-Plough, Kenilworth, NJ
IL-12	10 mg/ml	R&D Systems, Minneapolis, MN
IL-13	20 ng/ml	PeproTech EC Ltd., London, UK
GM-CSF	50 ng/ml	Dr. Glenn Begley, WEHI
TNF-α	100 ng/ml	R&D Systems, Minneapolis, MN

TGF-β	1 ng/ml	Dr. S. Chandler, LICR
Interferon (IFN)-α2a	1000 U/ml	Roche Products, DeeWhy, Australia
IFN-γ	1000 U/ml	Boechringer Ingelheim, Germany
PDGF	20 NG/ML	Dr. R. Whitehead, LICR
VEGF	10 ng/ml	Dr. S. Stacker, LICR
IGF-1 (CR3)	50 ng/ml	Dr. R. Whitehead, LICR
IGF-1 (PKC)	50 ng/ml	Dr. R. Whitehead, LICR
LIF	1000 U/ml	Dr. N. Nicola, WEHI
SCF	100 ng/ml	Dr. Glenn Begley, WEHI
FLT-3L	40 ng/ml	Genzyme Corp., Cambridge, MA
LPS: Sterotype 0111:B4	100 ng/ml	Sigma, St. Louis, MO
BSA	1%	Sigma, St. Louis, MO
Human Serum HS-LPS	10%	normal donors
(=LPS removed)	10%	normal donors; serum filtered with Zetapor filter (Cuno inc. Life Sciences Division, Meriden, CT) to remove LPS.

Cytokines were added on day 14 and cultures were
analyzed by flow cytometry for the presence of activated
(HLA-DR^{bright}, CD86$^+$) DC on day 17. Each set of experiments
(n=23) included control cells grown in standard conditions
5   in GM-CSF, TNF-α and IL-4 ("GTI"). The percentage of
activated DC in these control cultures on day 17 was
referred to as 100%. Standard deviations were calculated
for all experiments to control for the variations between
individual experiments. The results are summarized in
10   Figure 14, in which twice the mean of all SD was used to
define the no-difference interval (grey). Only normal
human serum (HS), human serum filtered through a Zetapor
filter to remove LPS (HS-LPS) and IFN-α2a were capable of
increasing the percentage of activated DC above 2 SD of the
15   control.

Cytokine Effects on the Late Phase of DC Differentiation
        If IFN-α was added daily between day 13 and
day 16, a significant increase in mature DC was observed
20   (n>10). CD34$^+$ progenitor cells were cultured in GM-CSF
(40 ng/ml), TNF-α (20 ng/ml) and IL-4 (500 U/ml) for
16 days and labelled with monoclonal antibodies against
CD11b and the DC associated markers CD83, CD86 and CMRF-44
prior to sorting. Flow profiles were gated for large sized
25   cells. A second group of cells was cultured under the same
conditions, but IFN-α (1000 U/ml) was added daily between
days 13 and 16. CD83, CD86 and CMRF44 expression was up-
regulated, whereas CD11b expression decreased. Since the
down-regulation of antigens might take longer than up-
30   regulation, IFN-α was added in 3 further experiments until
day 28. A similar percentage of large sized cells
expressed the antigens which up-regulate during DC
maturation (CMRF44, CD86). However, down-regulated
antigens (CD1a, CD11b) showed far lower expression in the
35   presence of IFN-α. Thus IFN-α accelerated DC maturation.

Example 10   Effects of Type I Interferons on DC
                    Maturation

                    To determine whether other type I interferons
could also accelerate DC maturation, IFN-α2a, IFN-α8 and
5   IFN-β were added to cultures containing GM-CSF, IL-4 and
TNF-α. All three interferons had similar capacity to up-
regulate HLA-A,B,C, CD80 and CD86 and to down-regulate CD1a
and CD11b expression on the large cell population (n=3).
These changes occurred within 3 days, and were
10   concentration dependent, in a range of 10-100 U/ml. As
shown in Figure 15. Furthermore, up-regulation of HLA-DR,
CD83 and CMRF44 expression was observed in response to the
IFN-α2a, as shown for CD83 in Figure 16. Cell numbers in
culture did not significantly change during the 3 days
15   exposure to IFN-α.

Example 11   IFN-α Activates DC Function
                    In order to examine whether IFN-α could also
activate DC function, the allostimulatory capacity of DC-
20 . containing bulk cultures was compared with or without
3 days exposure to IFN-α2a. Figure 18 shows the results of
6 individual experiments. Cultures were split on day 14
and IFN-α2a (1000 U/ml) was added daily for 3 days into one
half. Cultures exposed to IFN-α2a contained 23±3.4% CD86⁺
25   DC, and these cells showed significantly increased
allostimulatory capacity (*p<0.05), as shown in Figure 19A.
Cultures without IFN-α contained 9±1.5% CD86⁺ DC on day 17
(Figure 18). These results were confirmed by an MLR using
DC sorted on day 18. DC exposed to IFN-α 2a for 3 days
30   were more stimulatory than control DC, as shown in
Figure 25B.

Example 12   Effect of TNF-α and IFN-α
                    In order to investigate whether IFN-α could act
35   alone or required TNF-α to induce DC maturation, cultures
were washed on day 14 and continued until day 17 in serum-
free medium containing GM-CSF and IL-4 with or without TNF-

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α, IFN-α or both. Both TNF-α and IFN-α were required for
optimal maturation, as shown in Table 4. Thus, the
enhancement of DC activation by IFN-α under serum-free
conditions required the presence of TNF-α.

5

Table 4

Effects of TNF-α and IFN-α on DC Activation
Between Days 14 and 17

cytokine Antigen	DC as % of large cells		
	TNF-α	IFN-α	TNF-α + IFN-α
CD80	54 ± 7	44 ± 7	74 ± 5
CD83	50 ± 2	nd	72 ± 4
CD86	57 ± 10	47 ± 6	84 ± 2

10

Example 13   Type I Interferon Only Acts in Cultures
               Supplemented With IL-4
        Figures 17A and 17B show the effect of IFN-α
addition between days 13-16 on expression of CD80 and CD86
15 in DC cultures. Cells grown in GM-CSF (40 ng/ml) and TNF-α
(20 ng/ml) ("GT") did not up-regulate CD80 or CD86 in
response to IFN-α, whereas cells exposed to IL-4 for the
whole culture period ("GTI") responded better than cells
cultured in GT and IL-4 added only after day 10 (n=3).
20 This is consistent with the results presented in Example 5.

Example 14   IFN-α Production in Serum-Free Cultures of
               DC
        Since type I IFNs were the only cytokines that
25 could stimulate DC maturation, it appeared likely that
autocrine or paracrine production of IFN was responsible
for DC maturation in our serum-free cultures. This
hypothesis was tested by assaying culture supernatants for
IFN activity. Supernatants were collected at different
30 time between days 14 and 30. IFN activity corresponding to

12±2 IU/ml (range 8-25 IU/ml) was detected in 10 samples from cultures of 6 patients. In supernatants from cultures of 5 other patients, IFN-like activity was not detectable. These data suggest that type I interferons can be produced

5   by the cells in these cultures, and may act as autocrine or paracrine factors to regulate the final stages of DC maturation.

Since autocrine production of IFN-α can cause significant biological effects even in the absence of

10   detectable activity in the supernatant (Hamilton *et al*, 1996), we regard the presence of measurable activity in cultures from 6 out of 11 patients as significant. The clear effect of exogenously added IFN-α, the low level production of IFN-like activity in the cultures and the

15   prolonged period of immaturity even in the presence of high dose TNF-α all suggest that type I IFNs provide a necessary signal for the induction of DC maturation and activation.

Example 15   IFN-α Activates the Migration of Skin-
20   Derived DC
In order to determine whether this effect was specific for progenitor-derived DC *in vitro*, the effect of IFN-α2a on skin-derived DC was examined. Split skin samples were obtained from the Department of Plastic

25   Surgery, Austin and Repatriation Medical Centre, Melbourne. As shown in Table 5, in the presence of IFN-α (3000 U/ml), increased numbers of migrating dendritic cells were observed compared to those without IFN-α.

Table 5

Effect of IFN-α on Skin-Derived DC Migration

Protocol / time	Dendritic Cells x 10^3	
	24 hours	48 hours
X-Vivo20, no IFN-α	7 ± 2	13 ± 4
X-Vivo20 + IFN-α	44 ± 25	41 ± 13

Figure 20A shows the cumulative DC number of one representative experiment. DC migration was observed in 6 of 10 experiments. Furthermore, migrating DC exposed to IFN—α showed similar CD1a, CD83 and CD86 expression, but accelerated expression of CD80 compared to the cells which migrated in the absence of exogenous IFN—α (Figure 20B).

In addition to the effects of IFN-α on *in vitro* derived DC, we have shown that IFN-α activated migration of resident DC from split skin samples floating in serum-free medium. IFN-a accelerated CD80 expression on these cells similar to its effects on progenitor-derived DC. These results suggest that the adherent, immature DC in our serum-free cultures are similar to skin derived DC in response to type I IFN as well as in phenotype.

DISCUSSION

This study describes a method for growing mature and functionally potent DC from human CD34^{+} hematopoietic progenitor cells in the absence of serum. Three cytokines reported previously to support DC growth in the presence of serum (GM-CSF, TNF-α and IL-4) were studied.

The development of mature DC appeared to be a multistep process which took 3 to 4 weeks, resulting in >20% cells with typical dendritic morphology and phenotype. Key features were the very high expression of HLA-A,B,C and HLA-DR, expression of adhesion molecules (CD11a, CD11c, CD54, CD58), costimulatory molecules (CD40, CD80, CD86) and

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DC-associated molecules (CD83, CMRF44) (Zhou and Tedder, 1995; Hock et al, 1994; Fearnley, 1996), and absence of monocyte/macrophage associated markers (CD14, CD32). The expression of Birbeck granules in CD1a$^+$ DC suggests that a
5 Langerhans like cell is developing in these cultures. Although the antigen expression pattern of DC derived from CD34$^+$ cells in serum-free conditions is very similar to that of other DC types (Freudenthal and Steinman, 1990, Nestle et al, 1993), CD2 and CD5 are not expressed in our
10 cultures, whereas CD4 was expressed. In contrast, mature DC purified from peripheral blood expressed CD2 and CD5 but lacked CD4 (Zhou and Tedder, 1995; Wood and Freudenthal, 1992; O'Doherty et al, 1993; Zhou et al, 1992). It is possible that the expression of these markers is dynamic,
15 and reflects the history of cytokine exposure in the life of a DC. However, the alternative hypothesis that T cell markers like CD2 and CD5 might distinguish DC of different lineages has to be considered.

The cells raised in this study represented
20 terminally differentiated, functionally potent DC. In contrast to adherent PBMC, they did not transform into macrophages, but maintained CD83 expression when cytokines were removed. A similar behaviour of committed DC was recently reported in response to M-CSF (Szabolcs et al,
25 1996). Furthermore, these mature DC could be distinguished from macrophages because they were non-phagocytic (Sallusto and Lanzavecchia, 1994), and remained so in the presence of human serum.

Compared to autologous PBMC, DC-cultures were at
30 least 30 times more potent stimulators of allogeneic PBMC proliferation and of the expansion and activation of autologous, peptide-specific CTL. As has been described for cultures containing FCS (Strunk et al, 1996), IL-4 had a potent effect on differentiation of DC. We observed that
35 human serum inhibited DC differentiation, which underlines the value of this serum-free culture system obtaining DC under defined conditions. Foetal calf serum which is mainly

used by groups working with progenitor-derived DC contains
xenoantigens and the risk of introducing infectious agents
make these protocols less desirable for clinical studies.
Autologous human serum does not appear to be a better
5   alternative because of its adverse effects on DC
differentiation.

As described by others using serum-containing
conditions (Szabolcs et al, 1995; Caux et al, 1992;
Santiago et al, 1992; Reid et al, 1992), GM-CSF and TNF-α
10 were required for DC development under serum-free
conditions. Concurring with previous reports (Reid et al,
1992), TNF-α was necessary in the early culture period but
had an additional effect on optimal DC development beyond
the first 5 days of culture. IL-4 had a potent effect on
15 the differentiation pathway of DC, increasing CD1a
expression at day 14 and CD86 expression at day 28. When
IL-4 was added during the first week and removed after
day 7, the cultures already contained precursors committed
to a CD14$^-$ lineage that differentiated into CD83$^+$ DC.
20 Addition of IL-4 (after day 10) likewise decreased CD14
expression. This effect of IL-4 on CD14$^+$ cells is well
established (Romani et al, 1994; Ruppert et al, 1991;
Romani et al, 1990). Interestingly, in the absence of IL-4
a spontaneous reduction of CD14 expression occurred,
25 combined with acquisition of a mature DC phenotype. This
effect was suppressed by the addition of HS in the absence
of IL-4. A similar spontaneous maturation was previously
reported in the presence of FCS, but in this case,
bipotential CD14$^+$ cells had to be isolated from bulk
30 cultures before day 6 (Szabolcs et al, 1996). It is
possible that IL-4 is produced in serum-free cultures,
leading to the development of mature DC from CD14$^+$
precursors. The possibility of an IL-4 independent pathway
and the effect of early IL-4 addition to CD14$^-$ progenitors
35 and precursors can now be examined.

GM-CSF, TNF-α and IL-4 were necessary for DC
maturation, but did not appear to be sufficient for optimal

DC generation under serum-free conditions. Other factors must be important for both proliferation and maturation of these cells. In the presence of serum DC yields are 2-6 fold higher, suggesting that serum contains additional
5   DC growth and/or maturation factor(s). Secondly, a prolonged time course (21-28 days) was required for DC maturation when compared to previous reports, in which DC were generated in serum containing cultures after 6 days (Szabolcs et al, 1996). This permissive effect of serum on
10   DC growth in vitro was reported previously (Reid et al, 1992). Thirdly, the density dependence of DC production implies that cells in the culture may be providing additional autocrine or paracrine factors to enhance growth.

15   The time course of increase in cell numbers in serum-free cultures indicates the presence of proliferating CD33$^+$ progenitors in the serum-free cultures. This suggests that a more efficient expansion is possible. The early drop in viable cell number together with the
20   exclusive survival of CD33$^+$ cells suggests a selective cell death of non-myeloid cells.

            We investigated the effects of other cytokines on DC yields. As described by others, SCF was capable of expanding total cell yields without interfering with DC
25   development (Hamilton et al, 1996). This expansion, however, was less pronounced than that reported in previous studies in the presence of FCS (Hamilton et al, 1996). Since SCF is a growth factor with activity which is best seen in synergy with other haemopoietic factors (Molineux
30   et al, 1991), our results might be explained by the lack of additional serum factors. This also underlines the potential value of serum-free systems to study interactions of cytokines in defined conditions. Recently, another study was published using serum-free conditions to investigate
35   the effects of cytokines on DC produced from cord blood progenitor cells (Storbl et al, 1996). This study demonstrated that TGF-β could protect progenitor cells from

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apoptotic cell death (Riedl et al, 1997). Studies of TGF-β
with CD34$^+$ cells from adults have not been reported and in
our hands, TGF-β had only minimal effect on cell yields.
This may be due to the different progenitor sources in our
5   study.

Thus it has been shown that it is possible to
culture DC from CD34$^+$ progenitor cells under serum-free
conditions, and that these cells are potent stimulators of
autologous, peptide-specific, CD8$^+$ CTL. This method will
10 be useful to further characterize the growth and
differentiation factors involved in DC development (Romani
et al, 1990) and to study the phenotype of DC precursors.
Evidence is presented to show that IL-4 is produced in
these cultures and contributes to the maturation of CD83$^+$
15 DC.

The use of a novel serum-free culture system to
produce DC from progenitor cells has enabled us to
investigate the cytokines involved in DC maturation and
activation. The main findings reported here are the
20 accumulation of an immature DC population around day 14,
the prolonged, spontaneous maturation of this immature
population into activated DC during the following 14 days,
and the capacity of type I interferons to accelerate this
maturation within 3 days.

25   Three different pathways have been described
according to their intermediate ("early") stages which
result in functional DC of similar phenotype. These
include CD14$^+$, monocytic cells (Sallusto, Lanzavecchia,
1994; Sallusto et al, 1995), CD14$^-$, CD1a$^+$ Langerhans cells
30 (Frelinger et al, 1979; Katz et al, 1979) and CD14$^-$, CD1a$^-$
peripheral blood derived DC (Van Voorhis et al, 1982).
Under the conditions described here, CD34$^+$ cells
differentiated to an intermediate stage which is CD14$^-$,
CD1a$^+$, and positive for Birbeck Granules. This is
35 consistent with the Langerhans cell phenotype. As with
freshly isolated Langerhans cells (Williams et al, 1994),
these early DC in serum-free cultures were characterized by

intermediate HLA-A,B,C and HLA-DR expression, high
expression of CD1a, expression of CD11b and the lack of
accessory molecules (CD80, CD86) as well as the DC
associated molecules CD83 and CMRF44. In the presence of
5   TNF-α, these immature DC took another 14 days to acquire
phenotypic and functional characteristics which are typical
for activated DC. The phenotypic and functional changes
observed during this process were similar to those seen in
other studies (Teunissen *et al*, 1990; Yokozeki *et al*,
10   1996), and involved the up regulation of HLA-A,B,C, HLA-DR,
CD80, CD86, CD83 and CMRF44, down regulation of CD1a and
CD11b and functional maturation into highly allostimulatory
cells. In parallel, cells lost their ability to adhere to
plastic and became non-adherent, round cells with a corona
15   of thin dendrites.

        The spontaneous maturation in these serum-free
cultures suggested that the production of autocrine or
paracrine factors might be involved in this process. We
therefore screened a number of cytokines and other
20   molecules for their action in this system. Apart from
human serum, only type I IFNs (α or β) were capable of
accelerating maturation, so that within 3 days a majority
of the large sized cells in culture expressed CD80, CD83,
CD86 and started to down regulate CD1a and CD11b. This
25   effect of IFNs added into cultures containing GM-CSF, IL-4
and TNF-α was concentration dependent in a range between 10
and 1000 U/ml, and similar for three different type I IFNs
(α2a, α8 and β). In parallel with the phenotypic changes,
DC exposed to IFN-α had increased T cell stimulatory
30   capacity. Both IFN-α and TNF-α were required for DC
activation between days 14 and 17. Synergy of TNF-α and
IFN on HLA class I expression has been previously reported
at the level of transcriptional regulation (Johnson and
Pober, 1994). The nature of the effector in human serum
35   activating DC remains unknown. It has recently been shown
that human serum contains soluble CD14 which forms
complexes with LPS and as a complex can activate CD14⁻ DC

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(Verhasselt *et al*, 1997). Therefore, these results also
provide an explanation why LPS in the absence of serum did
not activate our CD14⁻ DC.

5   We have shown that IFN-like activity was produced
in most spontaneously maturing serum-free DC cultures.
Since autocrine production of IFN-α can cause significant
biological effects even in the absence of detectable
activity in the supernatant (Hamilton *et al*, 1996), we
regard the presence of measurable activity in cultures from
10   6 out of 11 patients as significant. The clear effect of
exogenously added IFN-α, the low level production of IFN-
like activity in the cultures, and the prolonged period of
immaturity even in the presence of high dose TNF-α all
suggest that type I IFNs provide a necessary signal for the
15   induction of DC maturation and activation.

In addition to the effects of IFN-α on in vitro
derived DC, we showed that IFN-α activated migration of
resident DC from split skin samples floating in serum-free
medium. IFN-α accelerated CD80 expression on these cells
20   similar to its effects on progenitor-derived DC. These
results suggest that the adherent, immature DC in our
serum-free cultures are similar to skin derived DC in
response to type I IFN as well as in phenotype.

Our results open a new perspective on the events
25   involved in DC maturation and activation, showing that type
I IFN can enhance the effect of TNF-α in the induction of
this process. This suggests that in addition to the
multiple immunomodulatory effects, type I IFNs may also
regulate immune responses at the level of the antigen-
30   presenting cell. Type I interferons have a well
established role in the response to infections with viruses
(Hertzog *et al*, 1991; van den Broek *et al*, 1995; Hwang *et
al*, 1995). Immunomodulatory effects include the promotion
of Th1 responses by inhibition of IL-4 and IL-5 secretion
35   (Demeure *et al*, 1994; Belardelli, 1995), increase in IFN-γ
producing cells (Brinkmann *et al*, 1993) and effects on IgG
production (Romani *et al*, 1989). Our studies show a novel

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mechanism, that type I interferons also mediate effects by
inducing maturation and activation of DC. This may help to
explain the autoimmune phenomena associated with the use of
IFN in hepatitis and cancer patients (Hertzog et al, 1991;
5   Ronnblom et al, 1991; Gisslinger et al, 1992; Preziati et
al, 1995). It may also provide an additional mechanism for
the anticancer effects of IFN-α in a variety of tumors
(Tsavaris et al, 1996; Kirkwood et al, 1997; Real et al,
1986; Foon, 1997; Sarna et al, 1987; Kaido et al, 1995).
10   Importantly, these results may have impact on
clinical strategies for developing cancer vaccines. While
interferons are being used as adjuvant therapy for cancer
and as anti-viral therapy, the effect of interferons on DC
function has not previously been defined. IFN-α is
15   therefore a useful candidate as a vaccine adjuvant in
clinical trials using tumor antigens as vaccines.
Furthermore, this serum-free system should assist the
further study of events associated with DC activation as
well as providing clinical opportunities using interferon-
20   activated DC as cellular adjuvants.
             For clinical applications, IFN-α may be used as
an adjuvant to anti-tumour vaccination strategies (eg. in
conjunction with peptides like MelanA, Tyrosinase, GP100
and the MAGE family, or together with autologous,
25   irradiated tumour cells with and without GM-CSF). The
intratumoural injection of IFN-α should be reconsidered,
since tumours contain DC which do not induce anti-tumour
responses. This may be because intratumoural DC are
inhibited from maturation and migration by tumour-produced
30   inhibitors (such as soluble neutralizing IFN-α receptors.
Such inhibitors could therefore be blocked by appropriate
agents, such as specific antibodies, so as to permit
maturation of these DC.
             In addition, autoimmune phenomenon are associated
35   with inteferon-α injections. This may likely be caused by
activated DC presenting self-antigens. The inhibition of

this pathological condition by inhibiting interferon may
therefore be considered.

5   It will be apparent to the person skilled in the
art that while the invention has been described in some
detail for the purposes of clarity and understanding,
various modifications and alterations to the embodiments
and methods described herein may be made without departing
from the scope of the inventive concept disclosed in this
10   specification.

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following pages, and are incorporated herein by this
reference.

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CLAIMS:

1.   A method of inducing the maturation of dendritic cells *in vitro*, comprising the step of culturing mononuclear cells in a serum-free medium in the presence of
5   a Type I interferon following an initial phase of growth in the presence of GM-CSF, TNF-α and IL-4.

2.   A method according to Claim 1 in which the mononuclear cells are obtained from a mammal which has been pretreated with a cytokine.

10   3.   A method according to Claim 2 in which the mammal has been pretreated with G-CSF, GM-CSF or Flt-3.

4.   A method according to any one of Claims 1 to 3 in which the mononuclear cells are CD34⁺ haematopoietic progenitor cells.

15   5.   A method according to Claim 4 in which the cells which are cultured are peripheral blood progenitor cells or bone marrow progenitor cells.

6.   A method according to any one of Claims 1 to 5 in which the cells to be cultured are enriched for CD34⁺ cells
20   prior to culture.

7.   A method according to any one of Claims 1 to 6 in which the Type I interferon is interferon-α, interferon-β, interferon-ω, or consensus interferon.

8.   A method according to Claim 7 in which the Type I
25   interferon is interferon-α2a, interferon-α8 or interferon-β.

9.   A method according to any one of Claims 1 to 8 in which the culture medium additionally comprises stem cell factor (SCF).

30   10.   A method according to any one of Claims 1 to 9 in which the cells are cultured for 14 to 35 days.

11.   A method according to Claim 10 in which the cells are cultured for 14 to 28 days.

12.   A method according to Claim 11 in which the cells
35   are cultured for 14 to 17 days.

13.   A method according to any one of Claims 1 to 12 in which the GM-CSF and TNF-α are present in the culture

- 55 -

medium during the whole period of culture, and the IL-4 is present commencing at day 6 or is present for the whole period of culture.

14.   A method according to any one of Claims 1 to 8 in which the Type I interferon is present in the culture medium commencing at day 13 of culture.

15.   A method according to any one of Claims 4 to 9 in which the SCF is present in the culture medium for the whole period of culture.

16.   A method of enhancing the antigen-presenting capacity of dendritic cells, comprising the step of exposing dendritic cells to a Type I interferon.

17.   A method according to Claim 16, in which interferon is administered to a recipient mammal *in vivo*, in order to activate the recipient's own resident dendritic cells, including Langerhans cells or other tissue antigen-presenting cells.

18.   A method according to Claim 16, in which antigen-presenting cells are infused or injected into the recipient, and interferon is sequentially or simultaneously administered systemically to the recipient mammal.

19.   A method according to Claim 17 or Claim 18 in which additional cytokines which induce proliferation of dendritic cells are administered sequentially or simultaneously to the recipient mammal.

20.   A method according to Claim 19 in which interferon is given sequentially or simultaneously with GM-CSF or GM-CSF and IL-4.

21.   A method according to any one of Claims 16 to 20 in which the Type I interferon is interferon-α, interferon-β, interferon-ω, or consensus interferon.

22.   A method according to any one of Claims 16 to 20 in which the Type I interferon is interferon-α2a, interferon-α8 or interferon-β.

23.   A method according to Claim 16 in which dendritic cells are subjected to maturation by the method of any one of Claims 1 to 15.

24.   A method of preparation of a cellular adjuvant
for treatment of a pathological condition, comprising the
steps of maturing dendritic cells *in vitro* according to the
method of any one of Claims 1 to 15, and exposing the
5   matured dendritic cells to an antigen associated with said
condition.

25.   A method according to Claim 24 in which the
condition is a neoplastic disease and the antigen is
derived from neoplastic cells.

10   26.   A method according to Claim 24 in which the
condition is a disease caused by an infectious agent and
the antigen is derived from the infectious agent.

27.   A method according to Claim 26 in which the
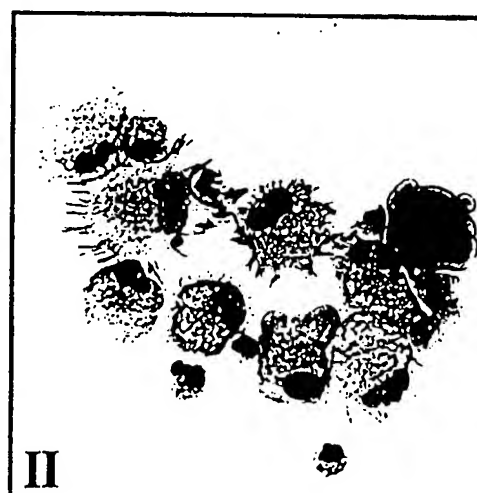infectious agent is a bacterium, a virus, a yeast or a

15   parasite.

28.   A method according to any one of Claims 24 to 27
in which the antigen is a protein, peptide, polysaccharide,
or nucleic acid.

29.   A method according to any one of Claims 24 to 28
20   in which the dendritic cells are transfected with the
antigen.

30.   A method according to any one of Claims 24 to 28
in which the dendritic cells are incubated *in vitro* with
the antigen.

25   31.   A method of producing activated T cells,
comprising the steps of harvesting dendritic cells and
lymphocytes from peripheral blood or bone marrow of a
patient suffering from a tumour, maturing dendritic cells
according to the method of any one of Claims 1 to 15, and
30   culturing the dendritic cells and the lymphocytes, either
separately or together, in the presence of an antigen, and
optionally in the presence of a cytokine.

32.   A method according to Claim 31 in which the
antigen is a tumour-associated antigen.

35   33.   A method according to Claim 31 or Claim 32 in
which the cytokine is a Type I interferon.

34.   A method according to Claim 33 in which the interferon is interferon-α, interferon-β, interferon-ω or consensus interferon.

35.   A method according to Claim 33 or Claim 34 in which the interferon-α is interferon-α2a or interferon-α8 or interferon-β.

36.   A population of mature dendritic cells which:

(a)   are non-phagocytic;

(b)   have potent antigen-presenting activity;

(c)   are capable of stimulating multiplication of allogeneic lymphocytes in the mixed leukocyte reaction (MLR),

(d)   are capable of stimulating autologous, peptide-specific cytotoxic T lymphocytes in the mixed leukocyte peptide culture assay system (MLPC), and

(e)   which are produced under serum-free conditions.

37.   A population of mature dendritic cells according to Claim 36 which strongly express MHC Class I and Class II antigens, CD80, CD83, CD86, and CMR44, and which either express CD1a and CD11b weakly or do not express CD1a and CD11b.

38.   Dendritic cell-activated tumour-associated T cells produced by the method of Claim 32.

39.   A cellular adjuvant produced by the method of any one of Claims 24 to 30.

40.   A method of treatment of a pathological condition, comprising the step of administering an effective dose of the cellular adjuvant according to Claim 39 to a mammal in need of such treatment.

41.   A method of treatment of a pathological condition, comprising the step of administering an effective dose of mature dendritic cells according to Claim 36 or Claim 37 to a mammal in need of such treatment, together with or subsequently to administration of an antigen associated with the condition.

42.   A method according to Claim 41 in which the condition is a neoplastic disease and the antigen is derived from neoplastic cells.

43.   A method according to Claim 41 in which the condition is an infectious condition.

44.   A method according to Claim 43 in which the condition is a disease caused by an infectious agent and the antigen is derived from the infectious agent.

45.   A method according to any one of Claims 41 to 44 in which the antigen is a protein, peptide, polysaccharide or nucleic acid.

46.   A method according to any one of Claims 41 to 45 in which the dendritic cells are the mammal's own cells.

47.   A method according to any one of Claims 42 to 46 in which the neoplastic cell antigen is from the mammal's own tumour cells.

48.   A method of treatment of a mammal suffering from a tumour, comprising the step of administering an effective dose of dendritic cell-activated, tumour-associated T cells according to Claim 38 to a mammal in need of such treatment.

49.   A method according to any one of Claims 42 to 48, in which the tumour antigen is a cancer-associated peptide such as Melan A, tyrosinase, GP100, an antigen of the MAGE family, a cancer testis antigen (CT) detectable by the SEREX method, or a member of the family of cancer antigens.

50.   A method according to any one of Claims 42 to 48, in which the antigen is derived from cancer cells or is autologous, irradiated tumour cells.

51.   A method according to any one of Claims 42 to 50 in which the antigen is administered in conjunction with GM-CSF.

52.   A method of using a Type I interferon as an adjuvant for peptide-based anti-tumour vaccination, comprising the step of administering a Type I interferon locally or systemically to a patient in need of such treatment, at the same time as or 1 to 3 days after

vaccination of the patient with a tumour peptide or tumour cell lysate, optionally in conjunction with GM-CSF.

53.   A method of using a type I interferon as an adjuvant for vaccination against an infectious agent, comprising the step of administering a Type I interferon locally or systemically to a patient in need of such treatment, at the same time as or 1 to 3 days after vaccination of the patient with the vaccine, optionally in conjunction with GM-CSF.

54.   A method according to Claim 53 in which the infectious agent is a bacterium, a virus, a yeast or a parasite.
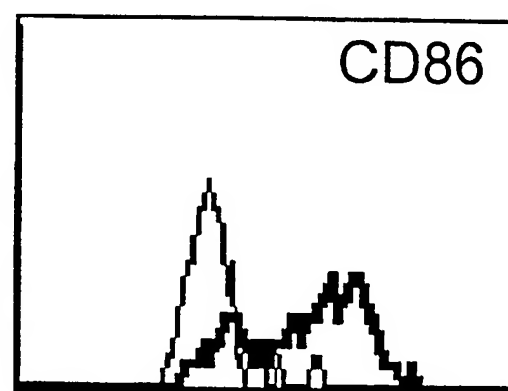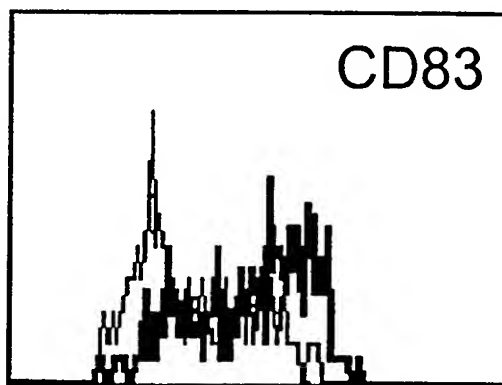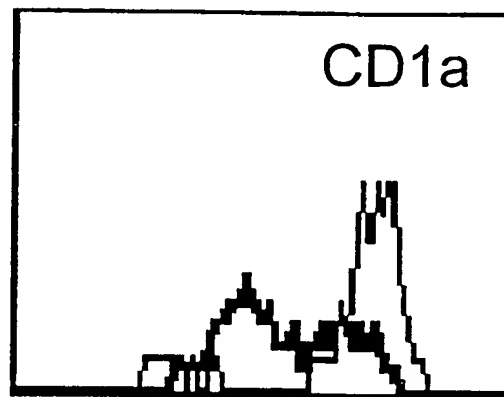
A)

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7

FIGURE 8

Up-regulated antigens

Down-regulated antigens

Fluorescence Height
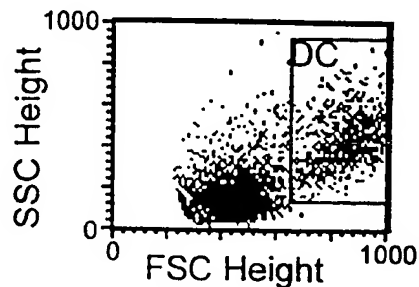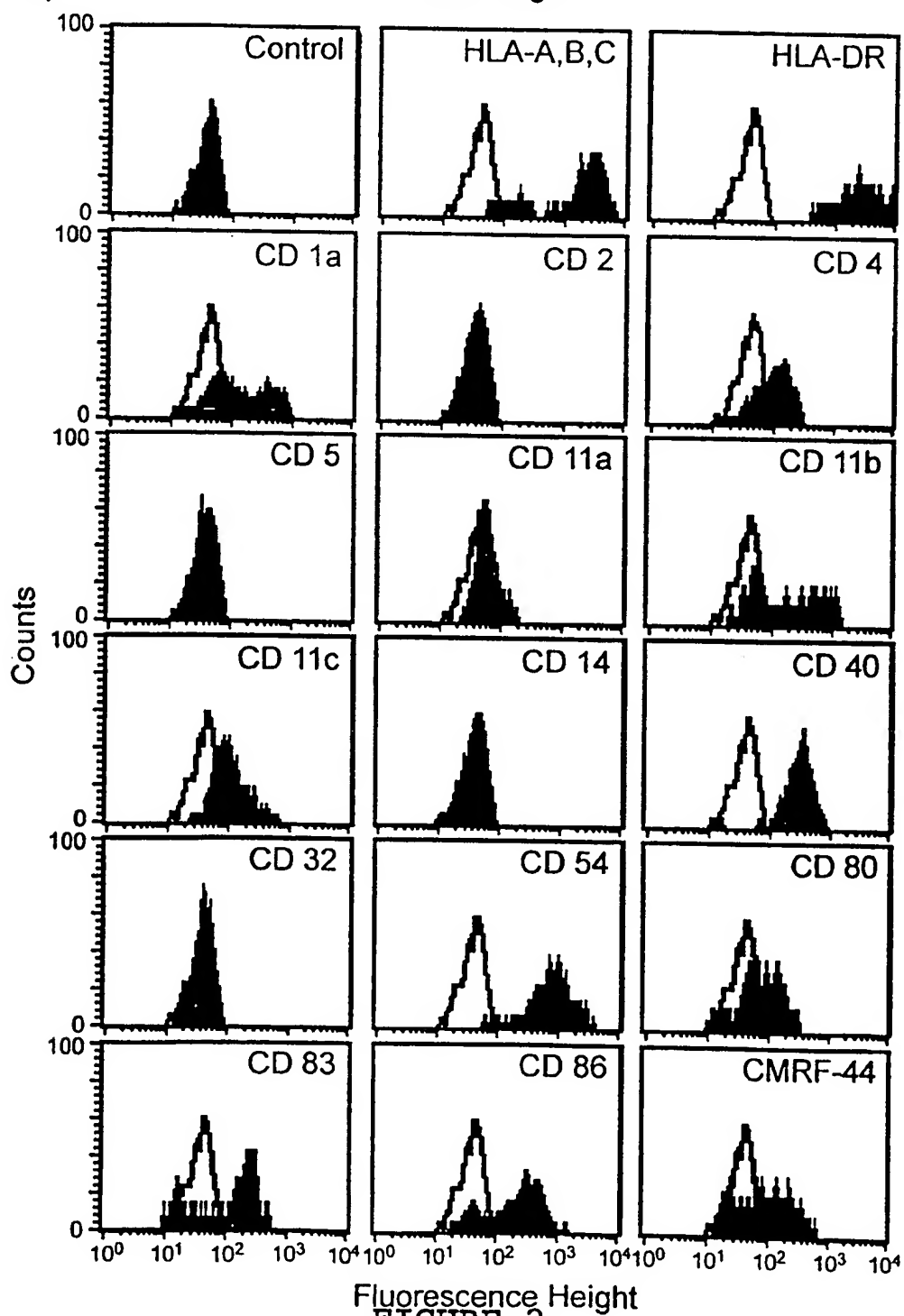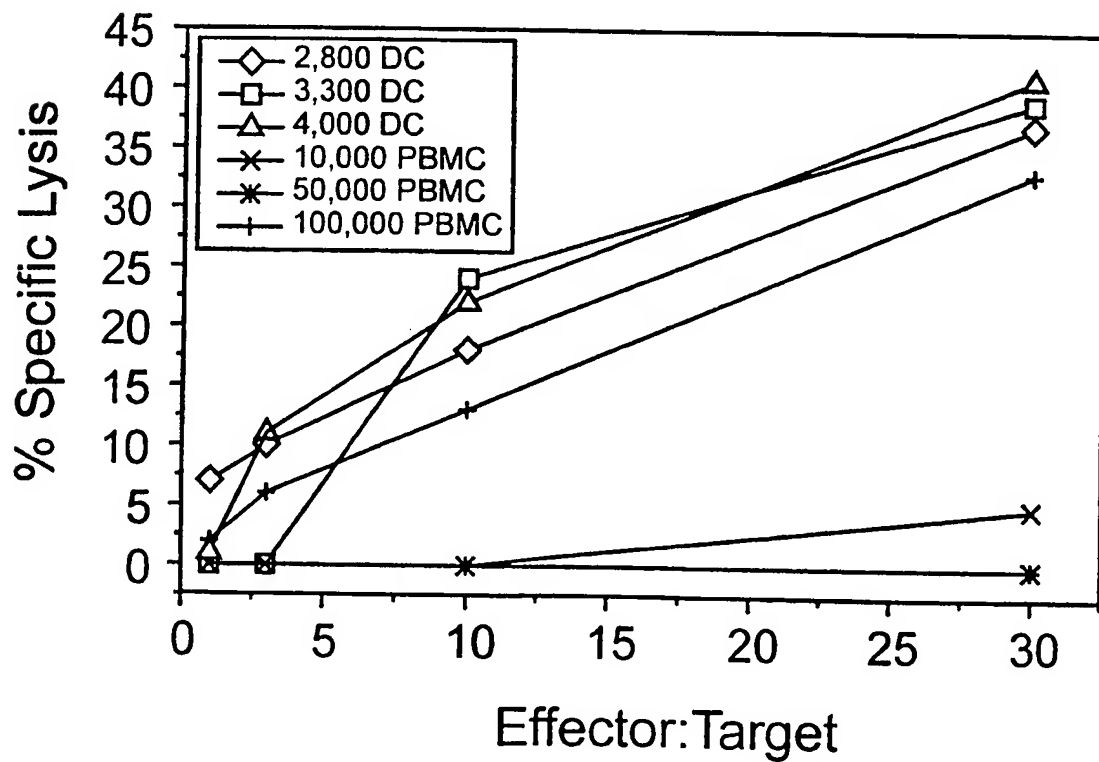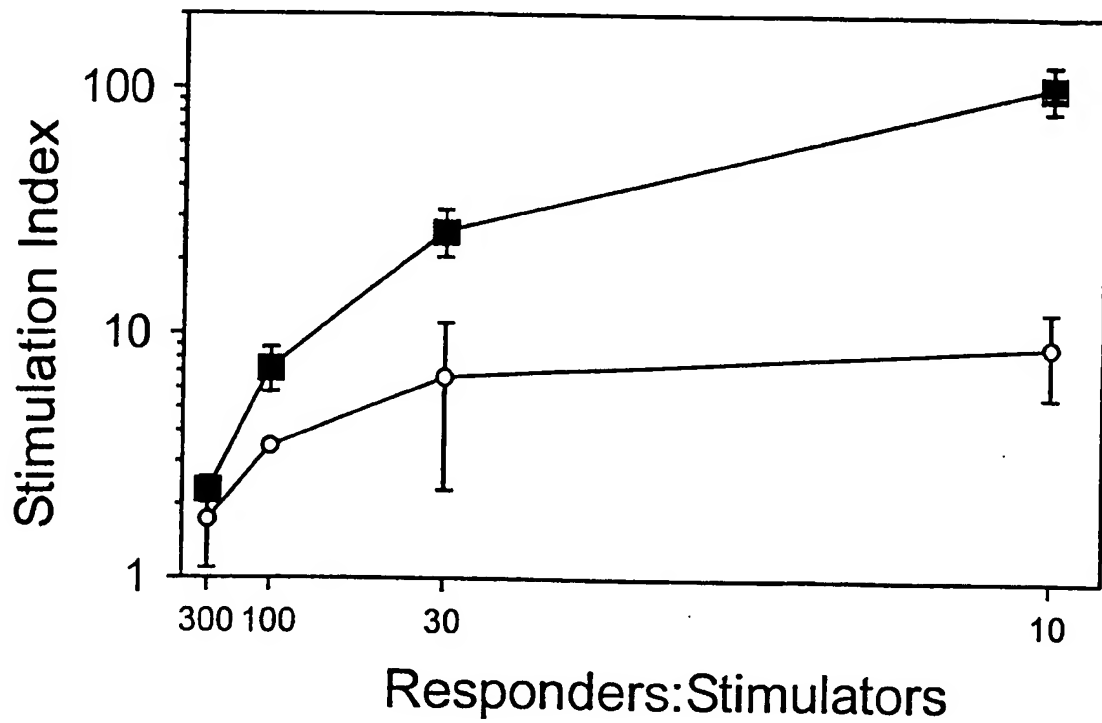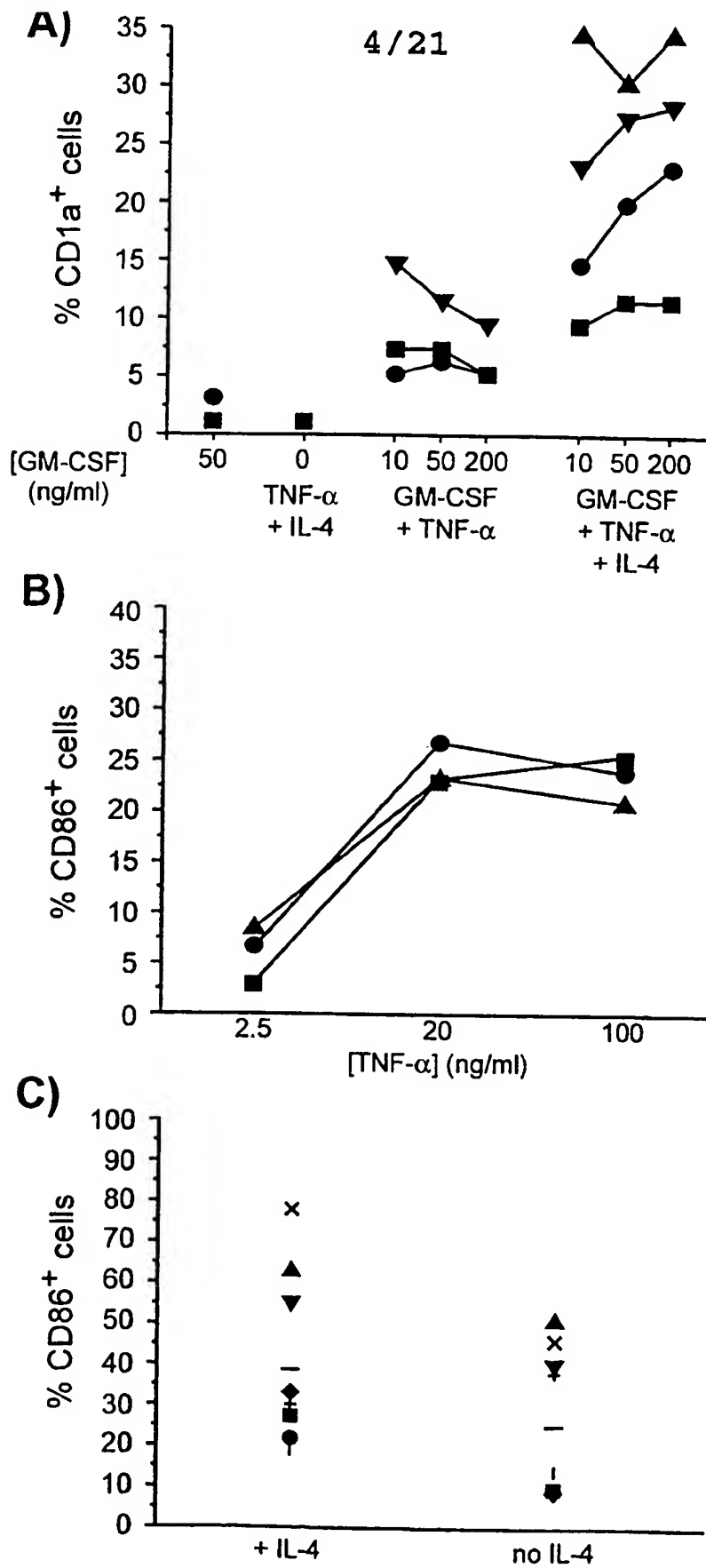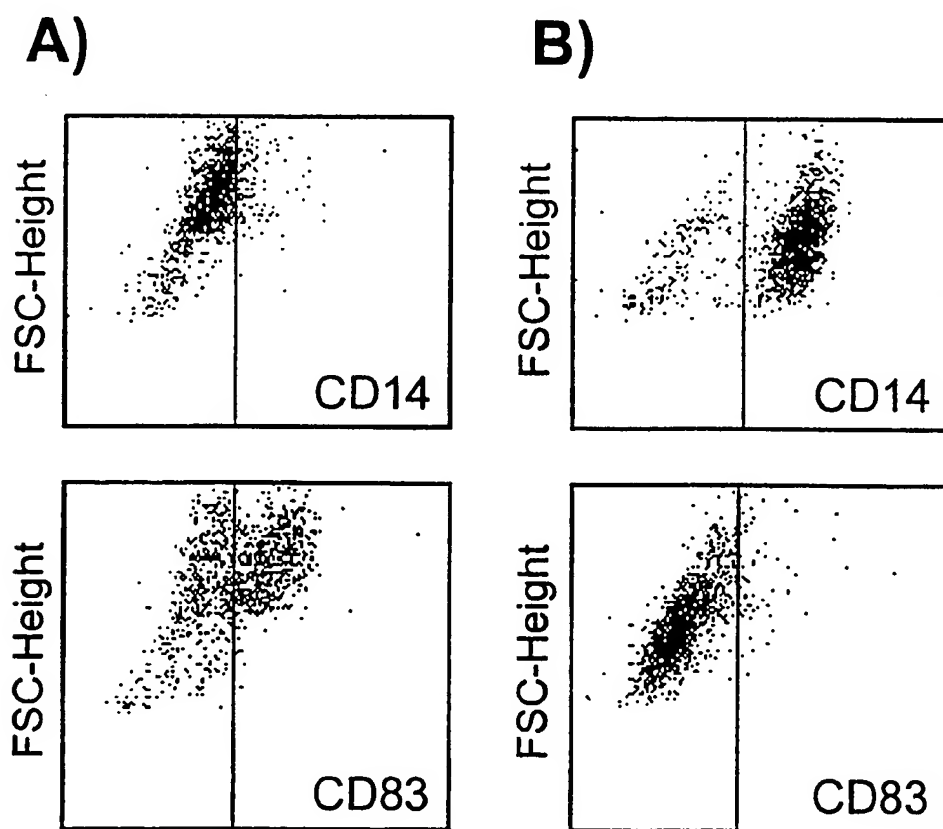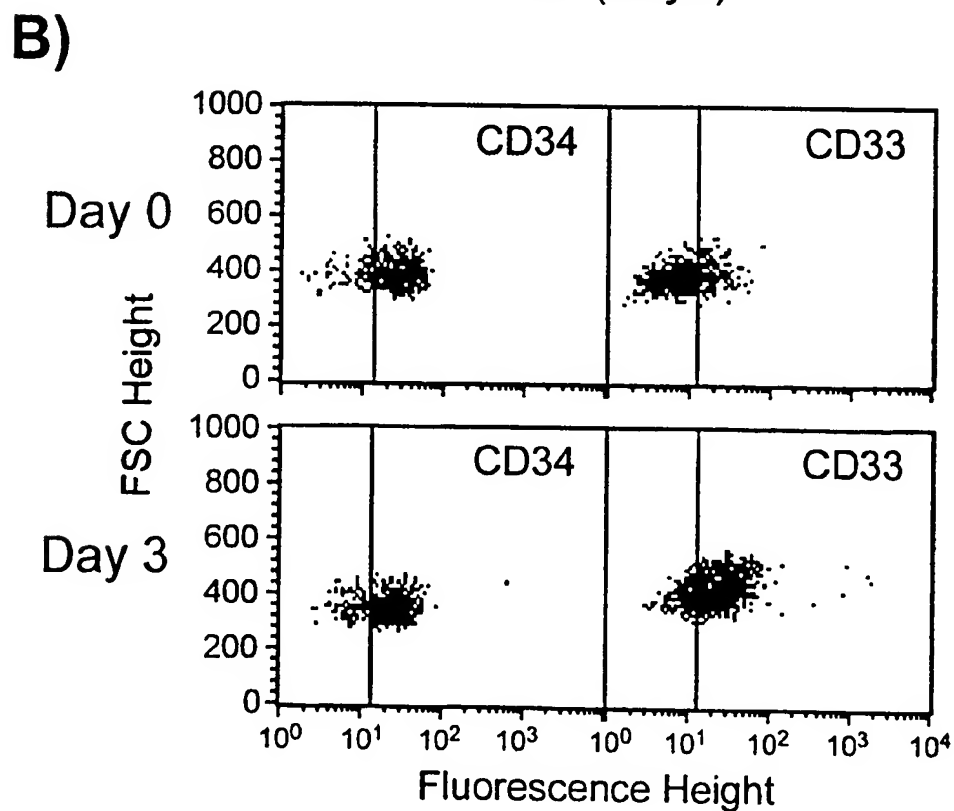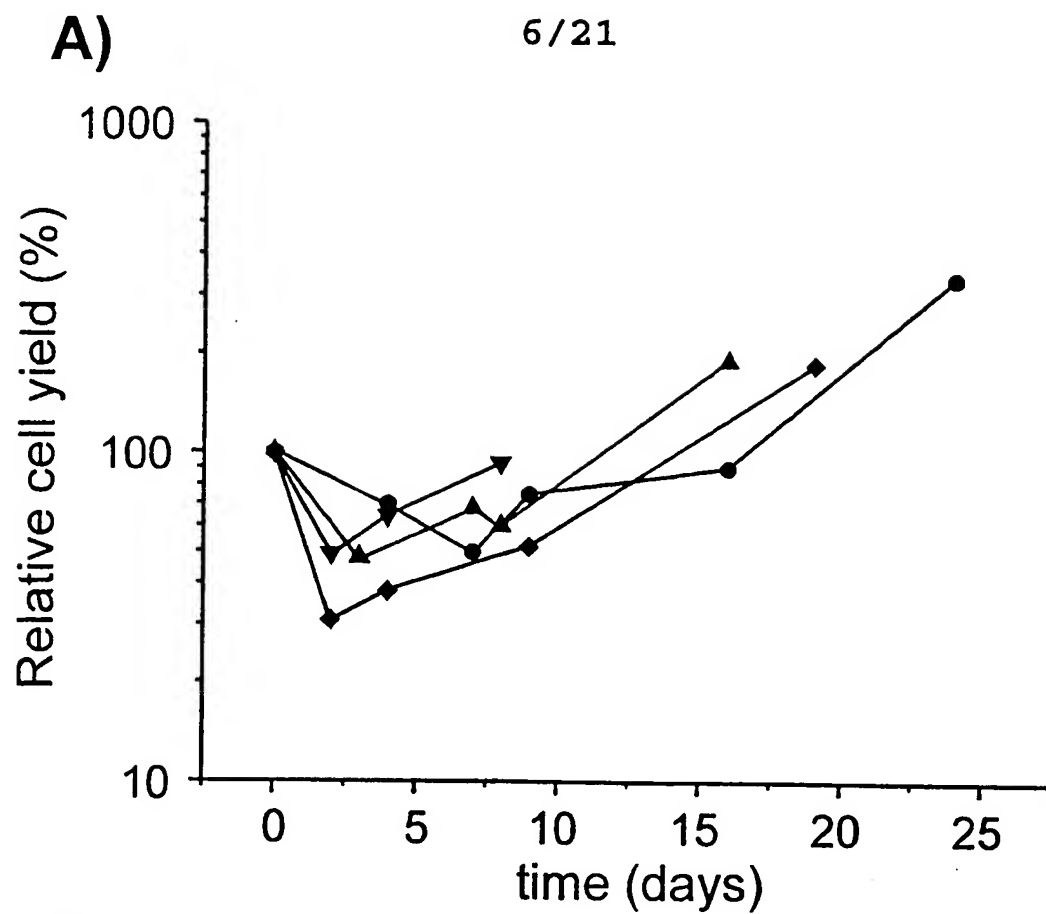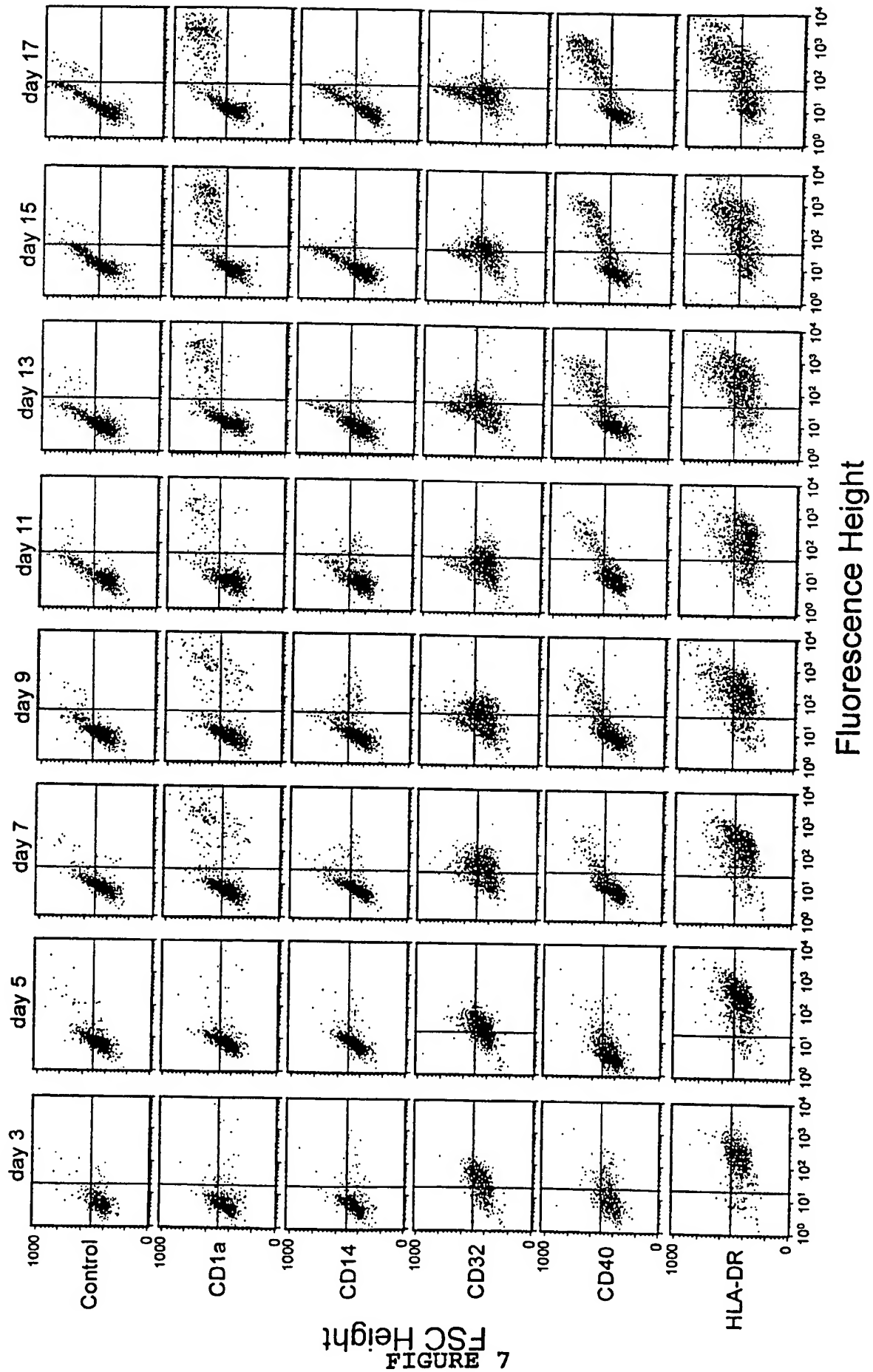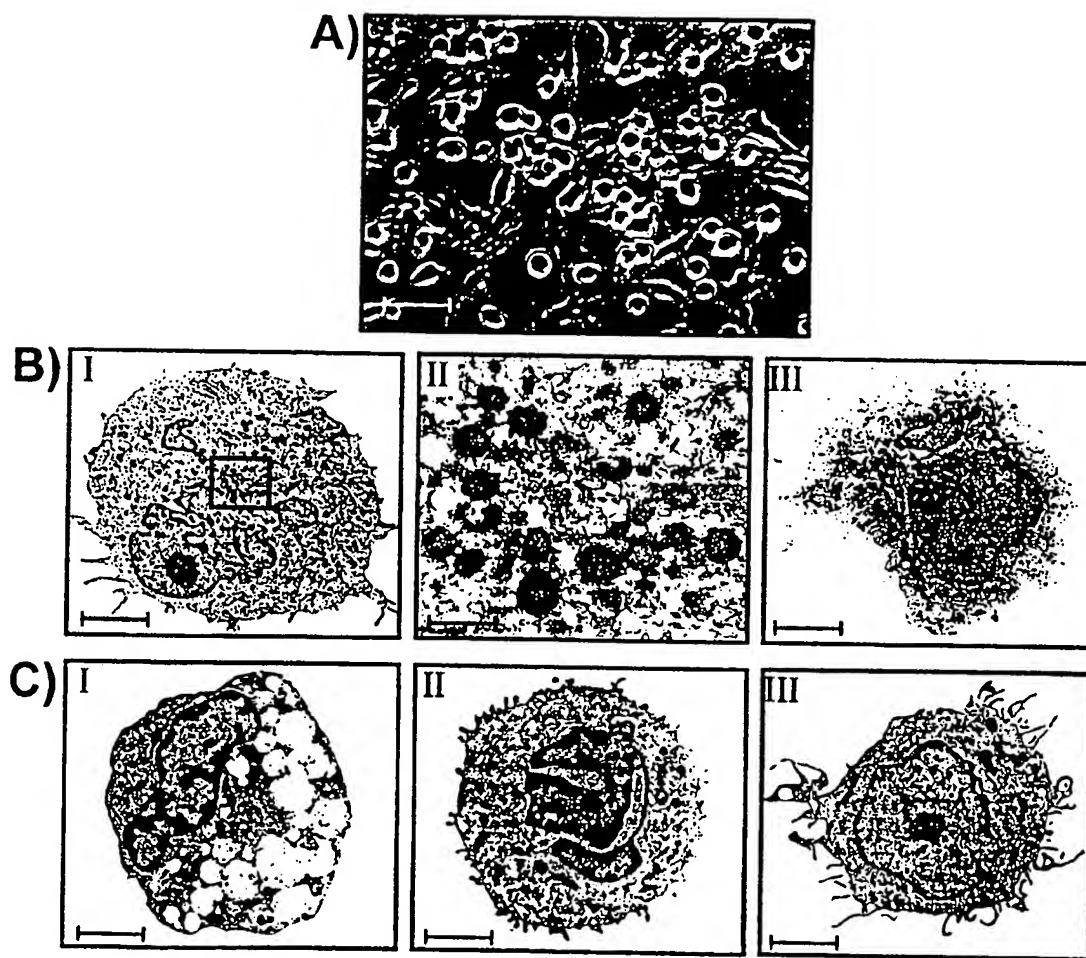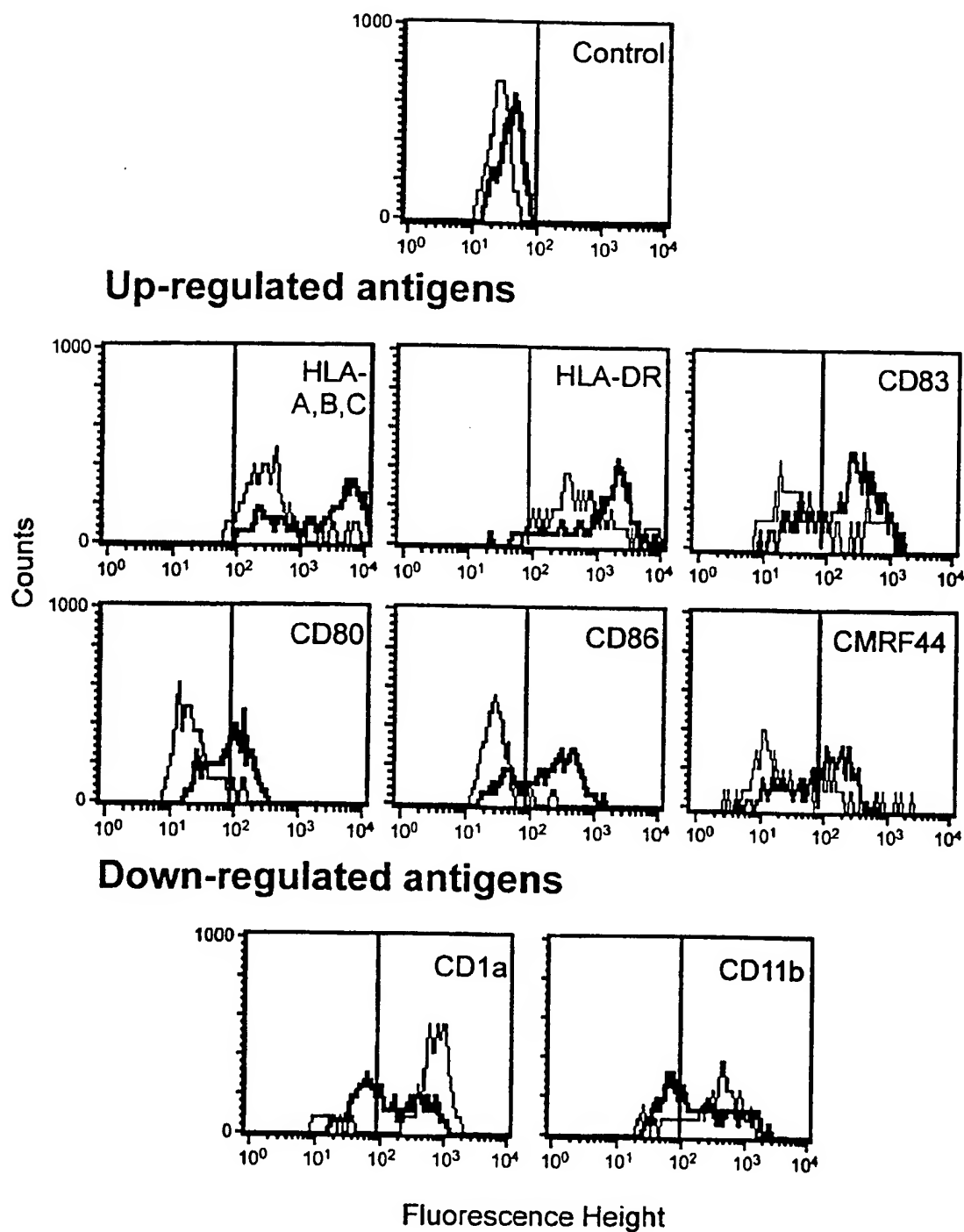
FIGURE 9A

FIGURE 9B

A)

B)

Fluorescence Height
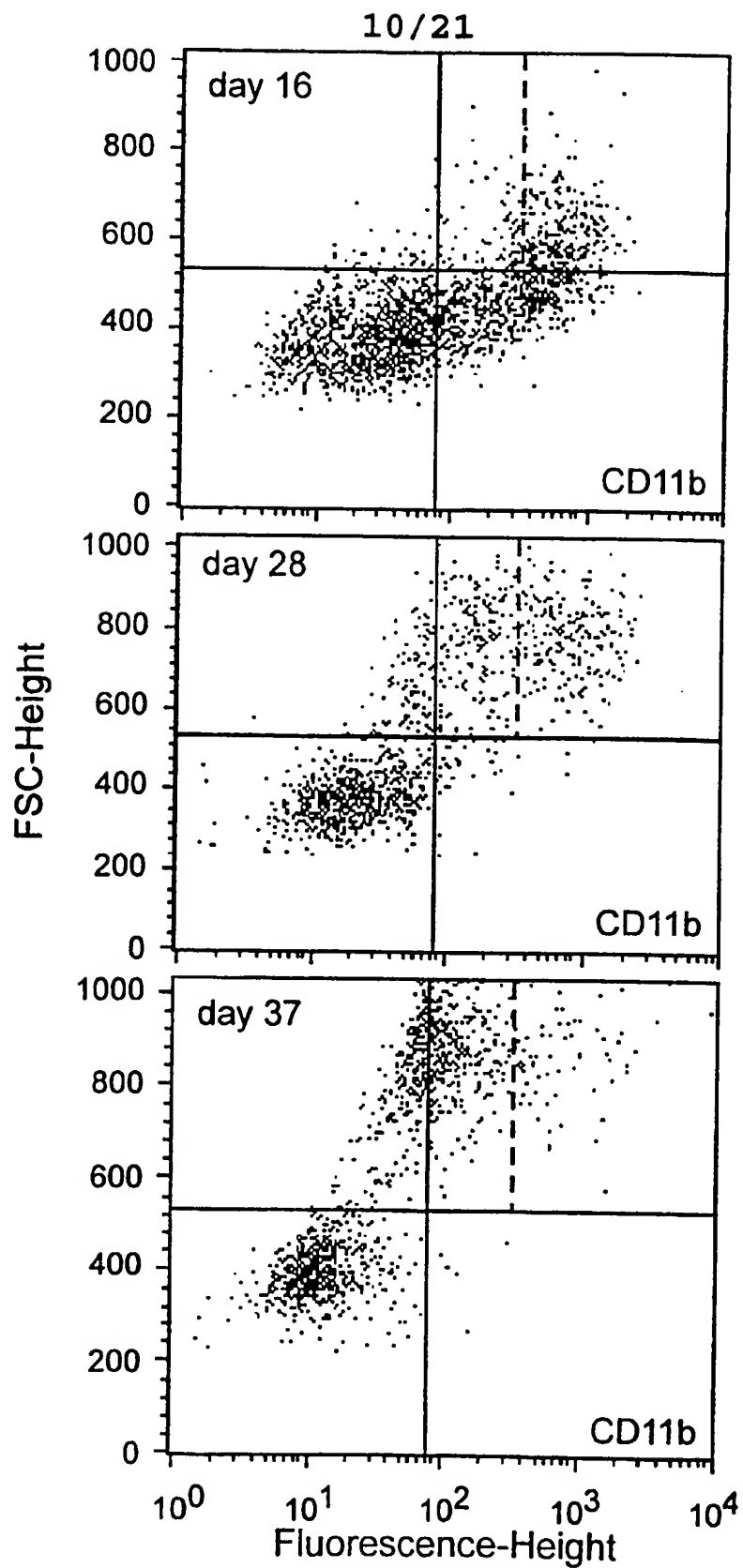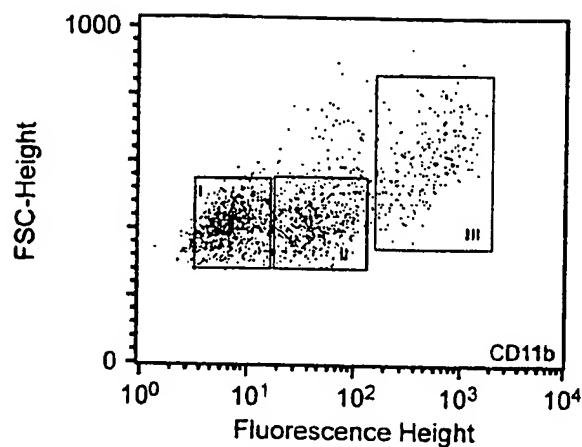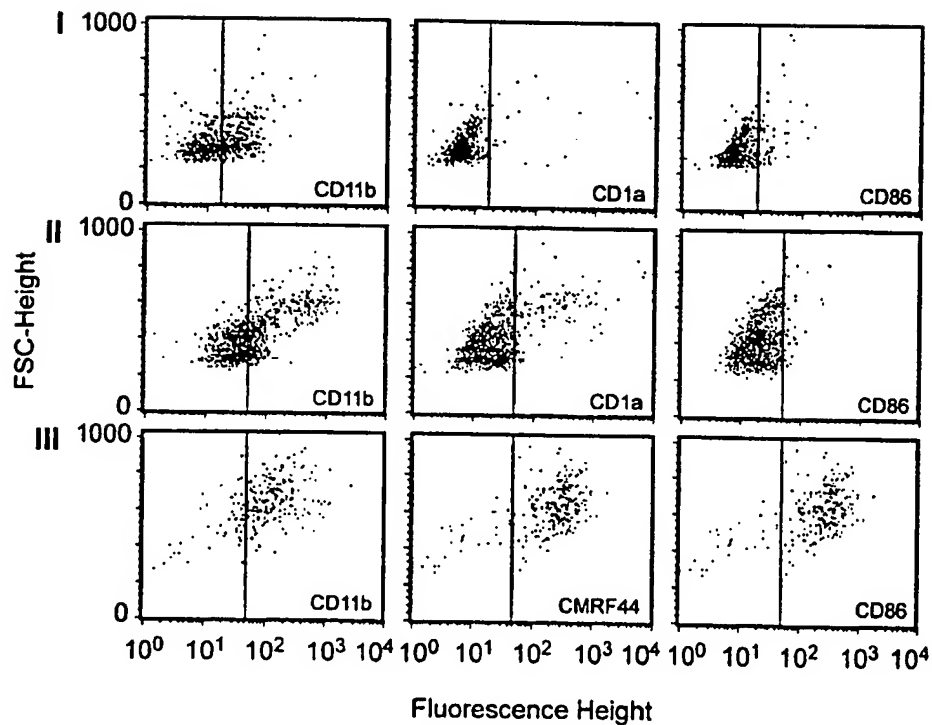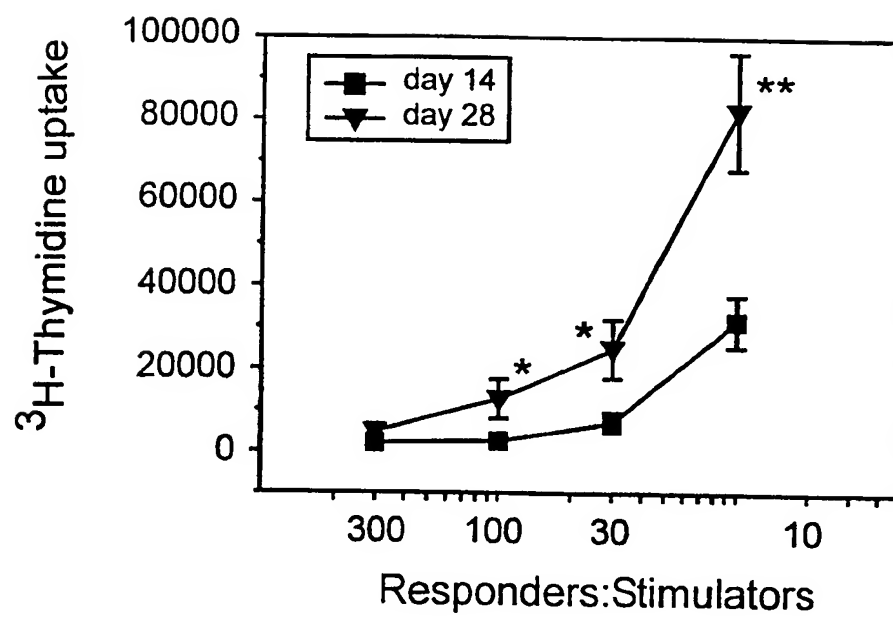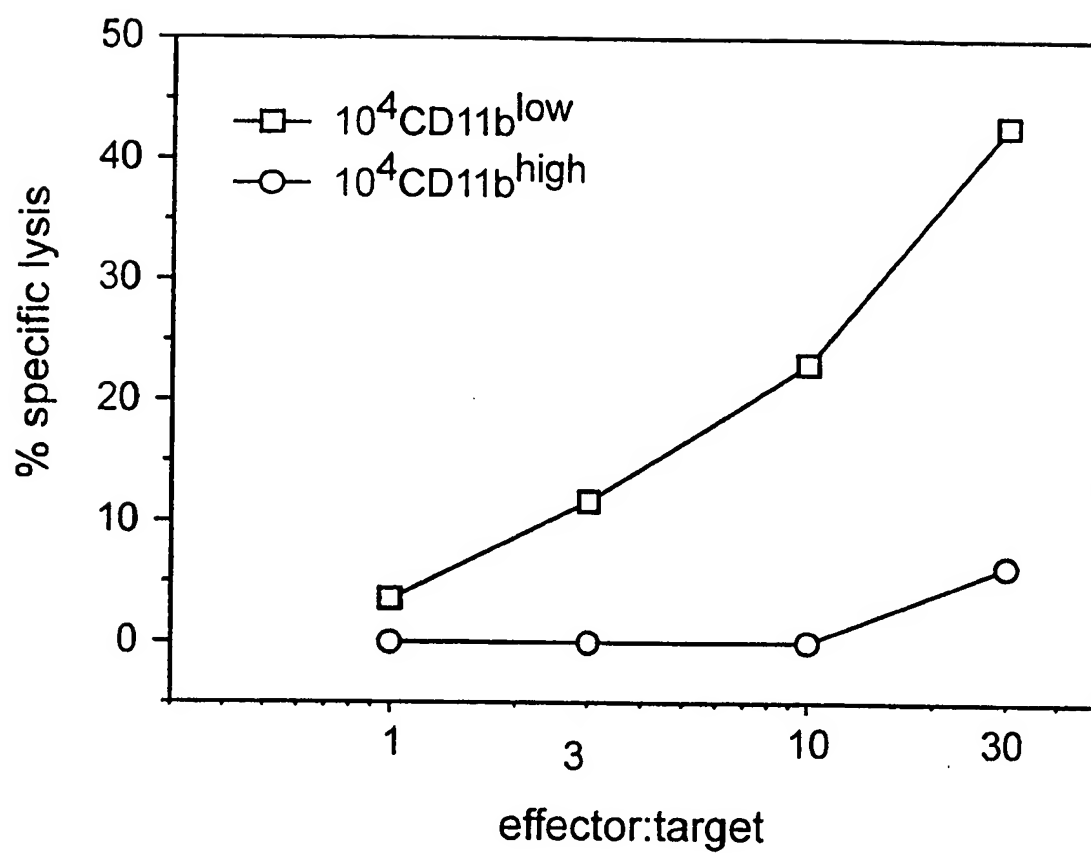
FIGURE 10

FIGURE 11

FIGURE 12
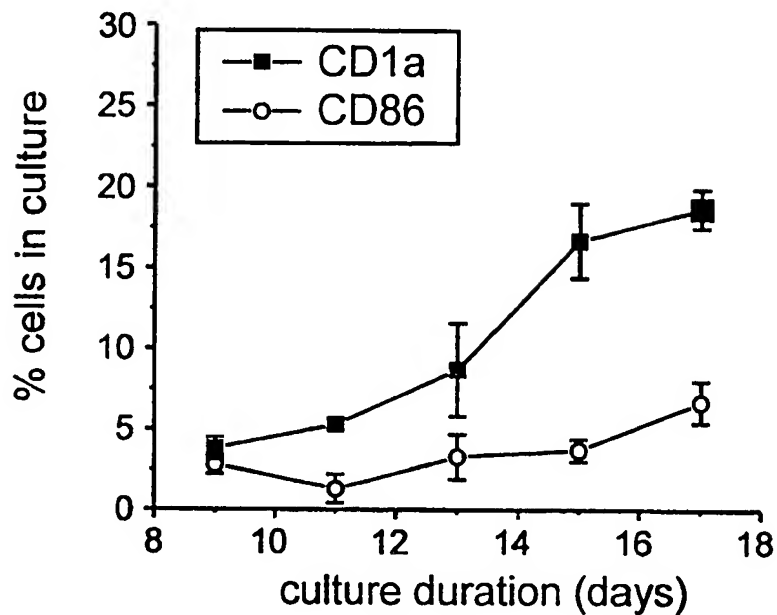
A) No human serum

B) + human serum
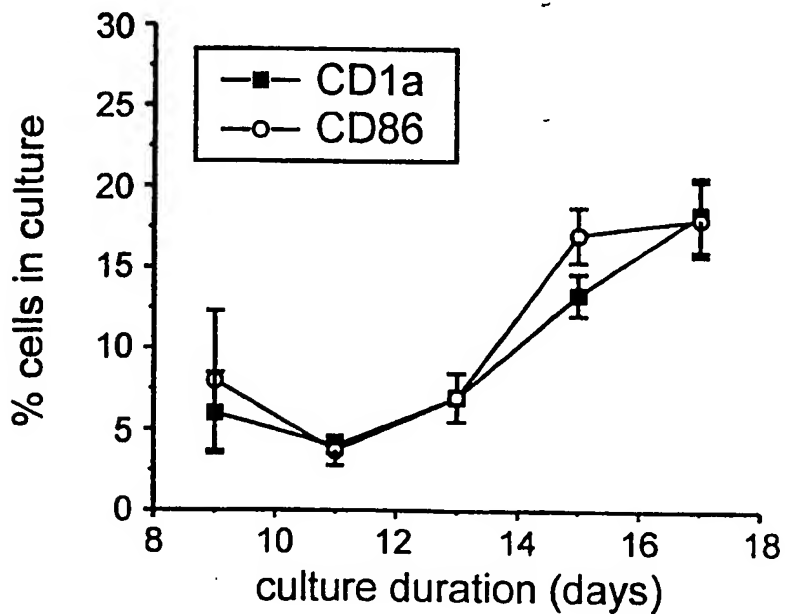
FIGURE 13

Relative Activated DC (%)
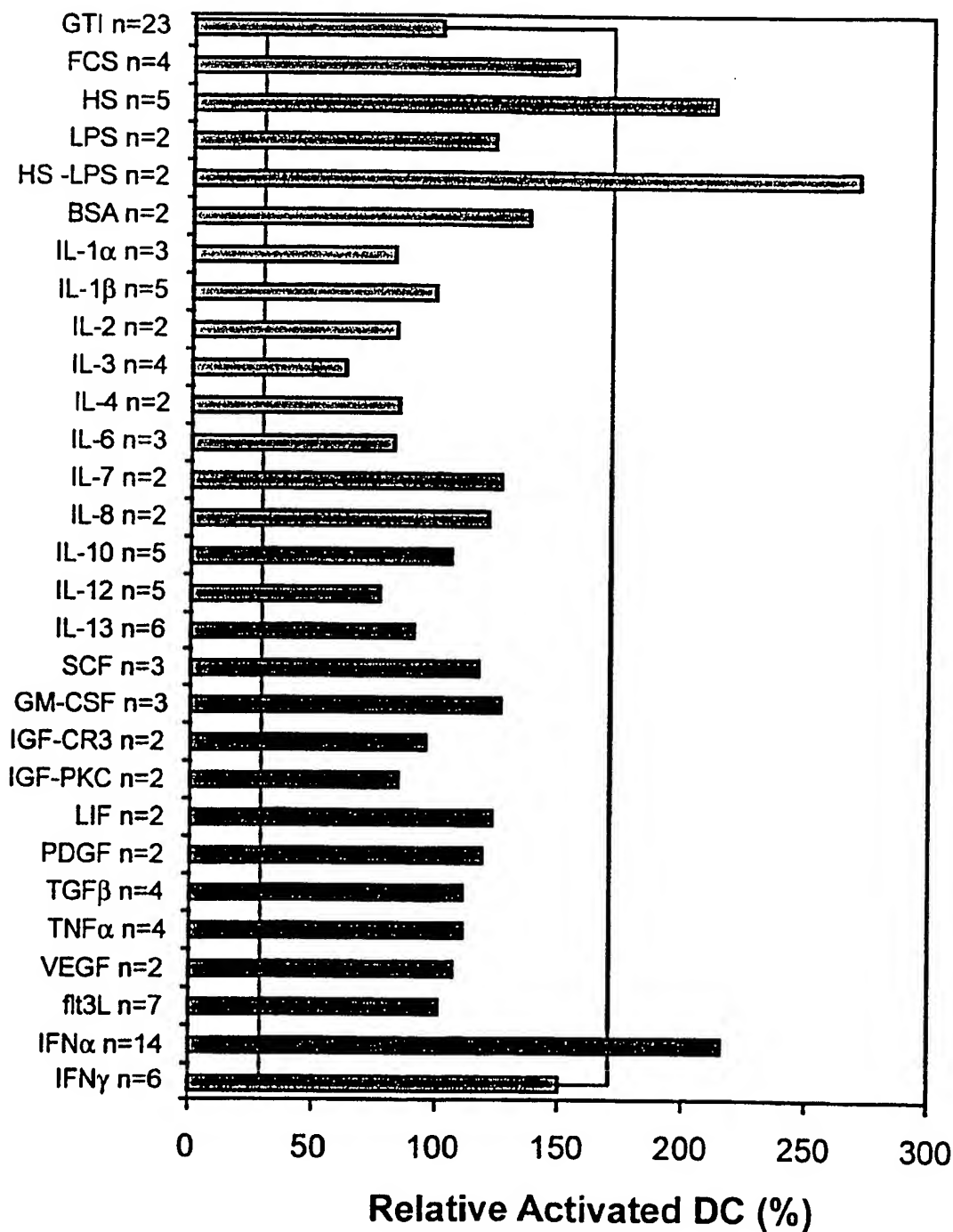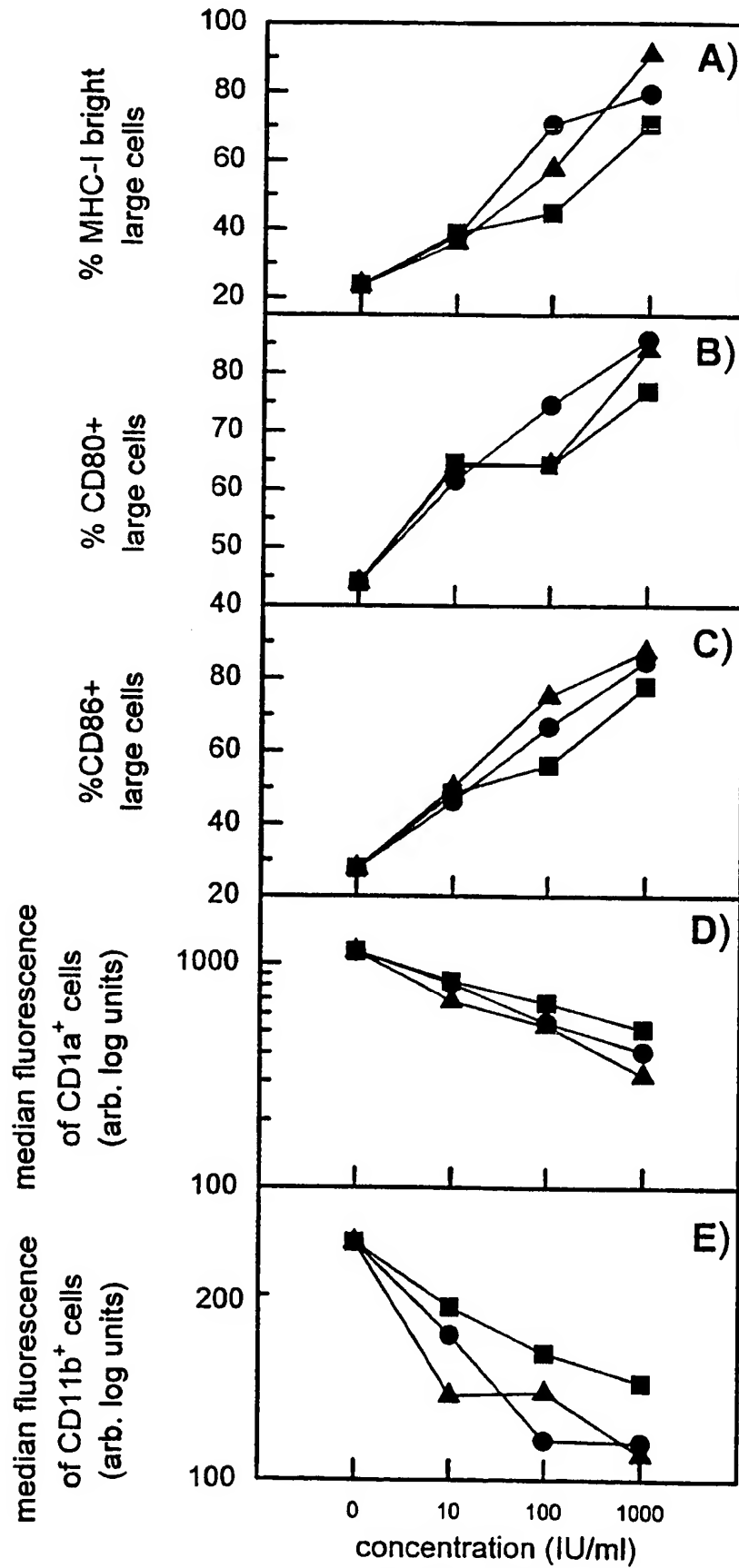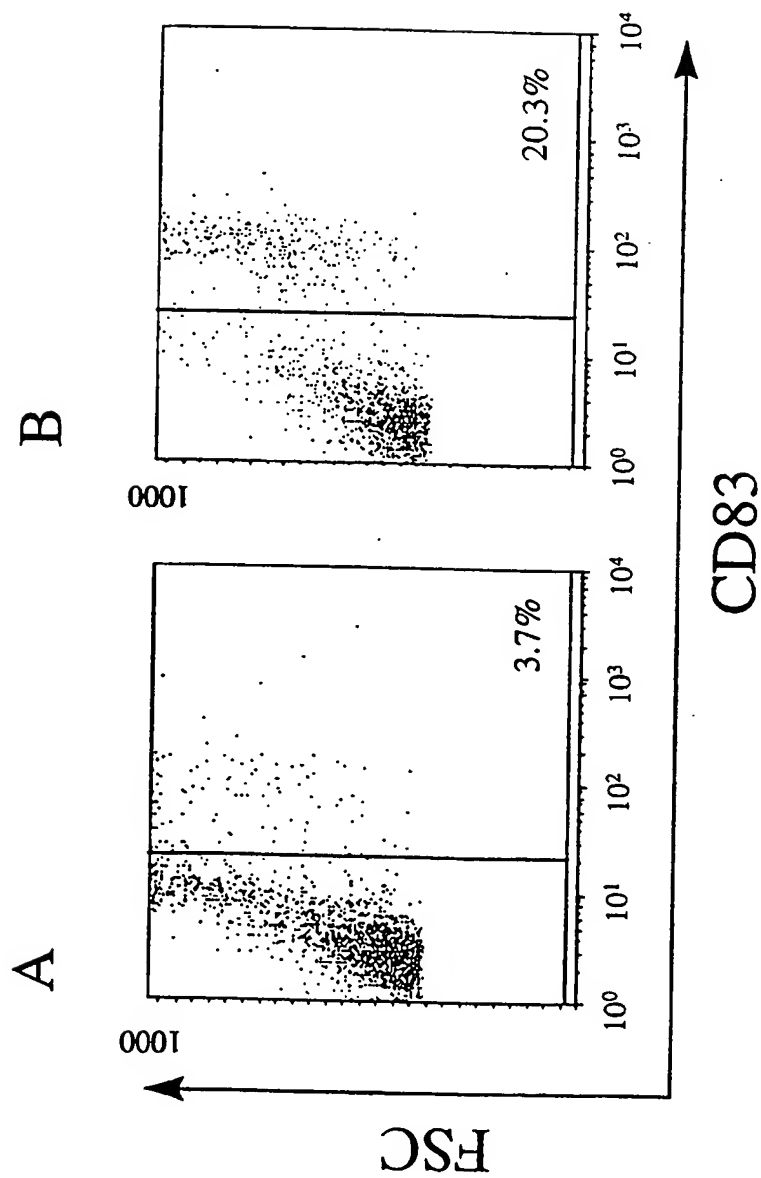
FIGURE 14
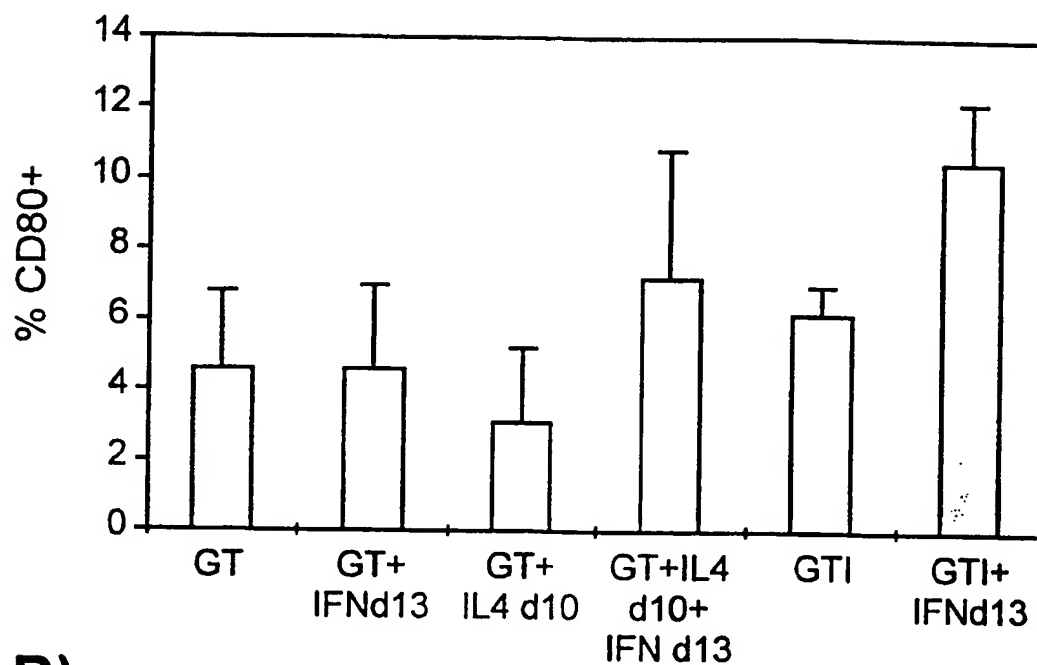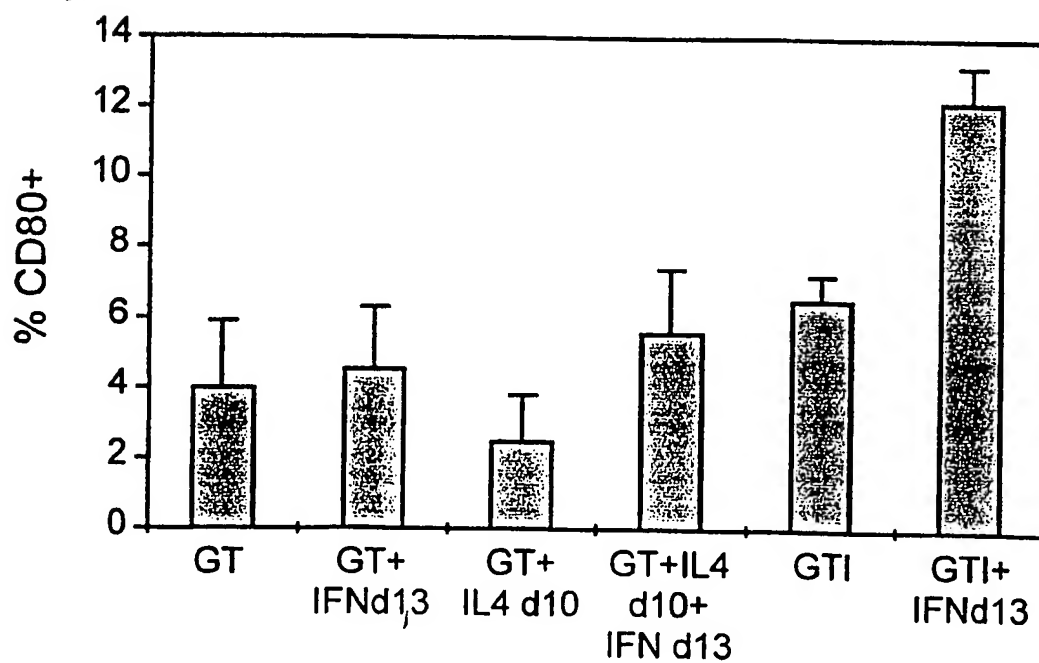
FIGURE 15

FIGURE 16

FIGURE 17

Phenotypic response to IFN-a
(n=6 cultures compared in parallel in the MLR)

IFN-a (1000 U/ml) was added to
half of the cultures from day 13 to 16
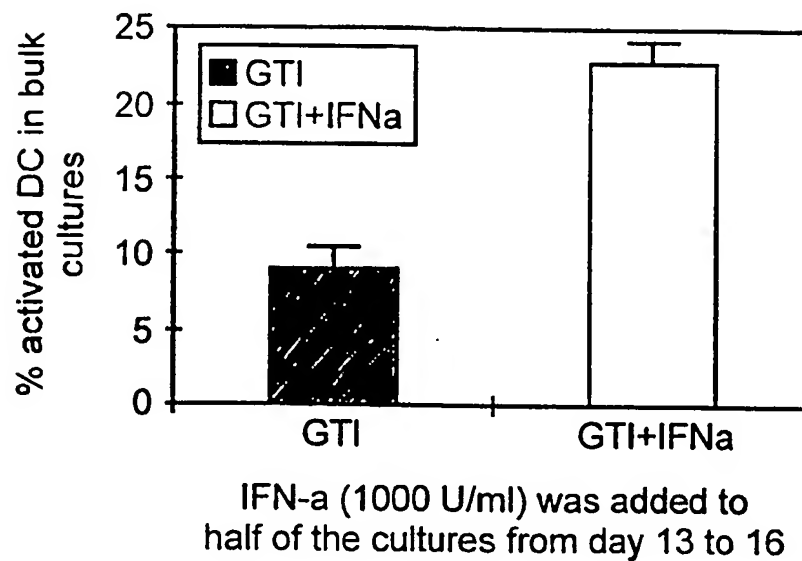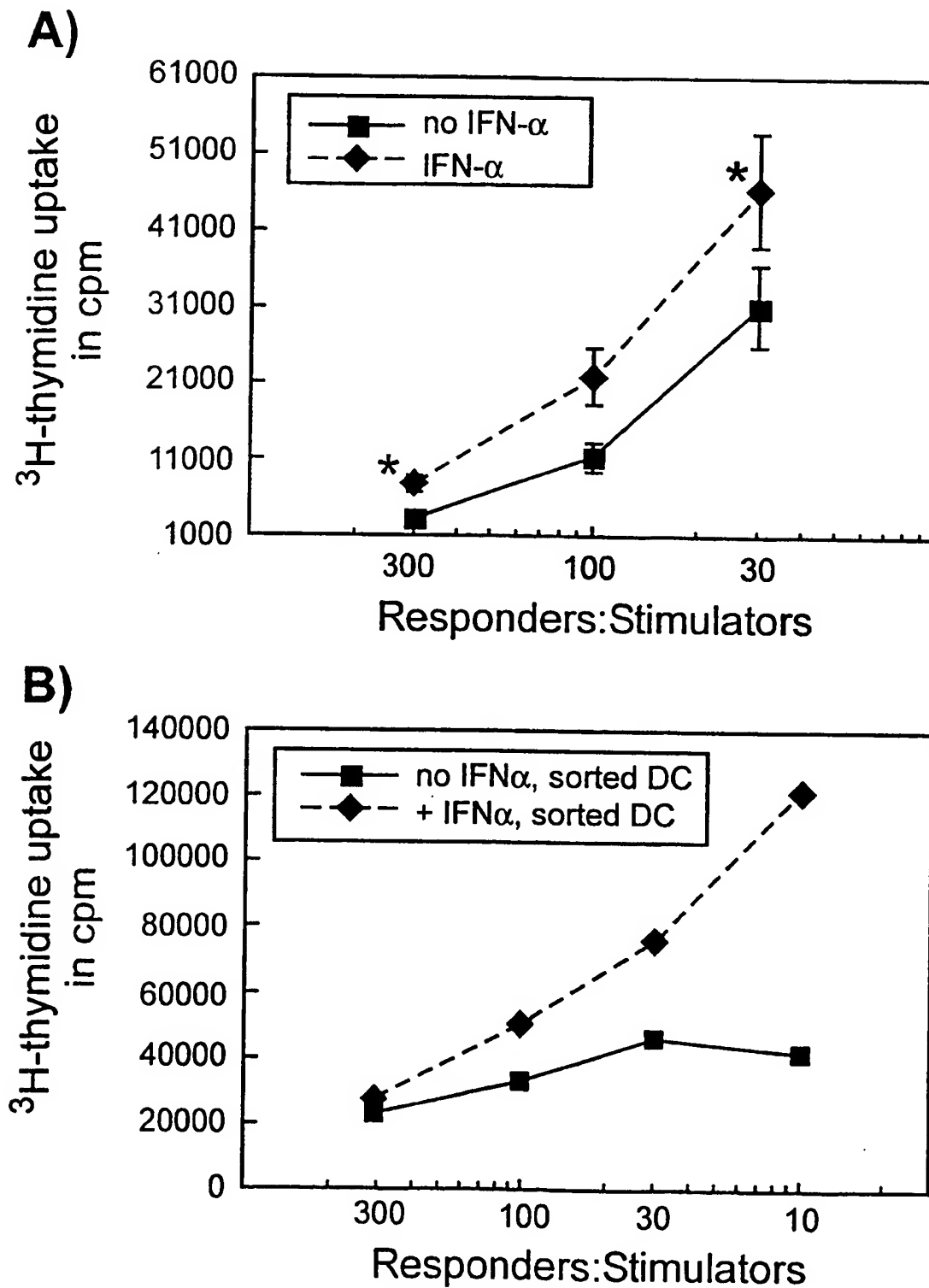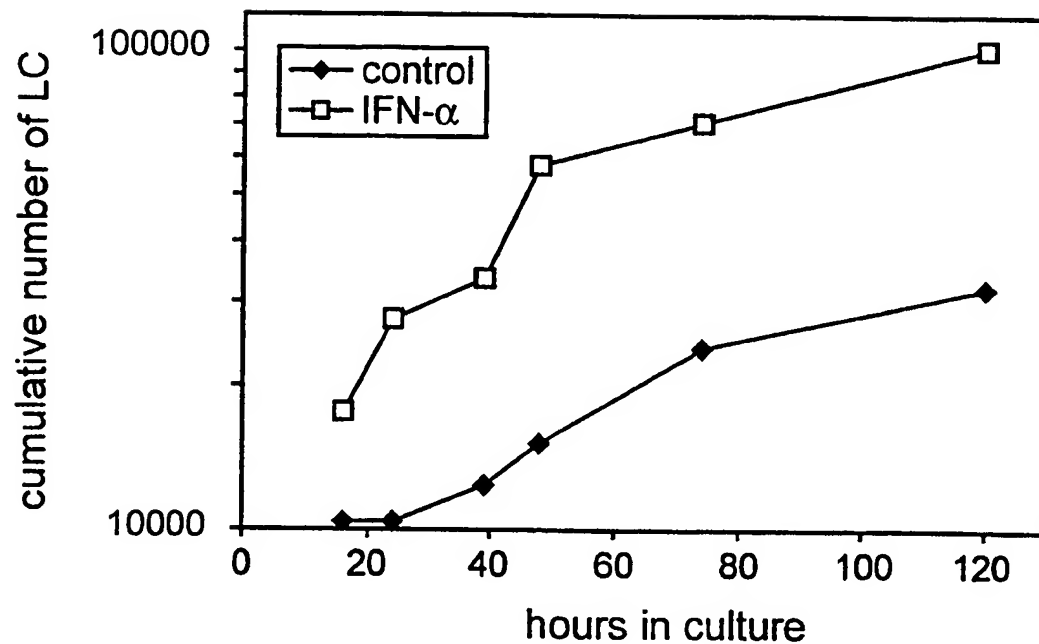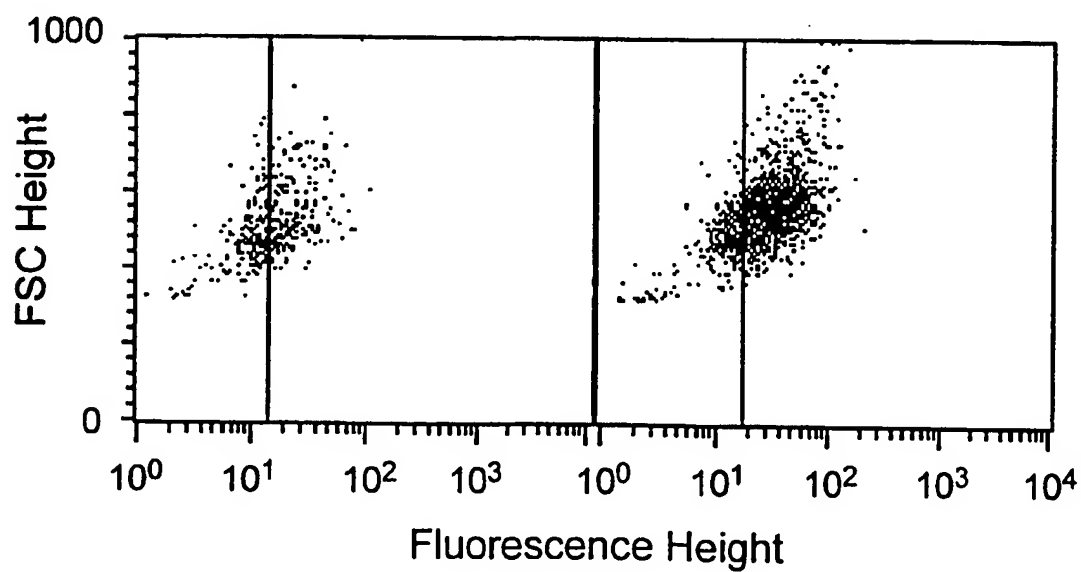
FIGURE 18

FIGURE 19

FIGURE 20

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00801

A.	CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]:   C12N 1/38, 5/02, 5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, CHEM ABS, MEDLINE: DENDRITIC CELLS AND GM-CSF AND TNF-ALPHA AND IL-4 AND IFN
(Also searched was the full forms of these abbreviations)

C.	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. of INTERFERON RESEARCH, vol. 10(4), 1990, pp 435-446, FELDMAN M and FITZGERALD-BOCARSLY P, "Sequential Enrichment and Immunocytochemical Visualization of Human-α-Producing Cells" Whole article	1-7, 16-39, 52-54
Y	ARCHIVES OF DERMATOLOGICAL RESEARCH, vol. 289, 1996, pp 1-8, JONULEIT H et al, "Cytokines and their effects on maturation, differentiation and migration of dendritic cells" Whole article	1-7, 16-39, 52-54
Y	J. EXP. MED., vol. 179, April 1994, pp 1109-1118, SALLUSTO F & LANZAVECCHIA A, "Efficient Presentation of Soluble Antigen by Cultured Human Dendritic Cells Is Maintained by Granulocyte/Macrophage Colony-stimulating Factor Plus Interleukin 4 and Downregulated by Tumor Necrosis Factor α" Whole article	1-7, 16-39, 52-54

[X]	Further documents are listed in the continuation of Box C	[X]	See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 December 1997	06 JAN 1998

Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer BARRY SPENCER Telephone No.: (02) 6283 2284

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 97/00801

C (Continuation)	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	P.N.A.S. USA, vol. 93, March 1996, pp 2588-2592, ZHOU LJ & TEDDER TF, "CD14⁺ blood monocytes can differentiate into functionally mature CD83⁺ dendritic cells" Whole article	1-7, 16-39, 52-54
P,Y	AU, A, 73922/96 (IMMUNEX CORPORATION) 10 April 1997 Claims 1-19	1-7, 16-39, 52-54

INTERNATIONAL SEARCH REPORT
Information on patent family members

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
AU	73922/96	WO	97/12633

END OF ANNEX